Investigating the role of selected epigenetic silencing marks in determining mouse skeletal muscle oxidative phenotype

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Abstract

Epigenetic regulation of gene expression supports skeletal muscle phenotypic plasticity. The role of epigenetic silencing marks in maintaining muscle phenotype and in supporting muscle plasticity remains unknown. This study aims to characterize whether silencing marks established by Mdm2 and EZH2 play a role in determining the muscle oxidative phenotype at rest and after training. In sedentary C57Bl/6 female mice, the soleus muscle showed higher protein levels of Mdm2 and H3K27me3, and lower levels of EZH2 compared to glycolytic muscles. 9 weeks of endurance exercise training changed the protein levels of Mdm2, EZH2, and H3K27me3 in a muscle-specific manner in C57Bl/6 mice. Differentiation of C2C12 myoblasts depleted EZH2 protein levels, and increased the protein levels of Mdm2 and H3K27me3. Overall, our study shows that phenotypically different muscles show a different relative abundance of silencing marks. Endurance training may also affect the presence of silencing marks.

Dedication

This thesis is dedicated to my wonderful parents and sister who have given me endless love, encouragement, and support through the good times and the bad times. Without you, I would not be where I am today. Thank you for everything.

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List of abbreviations

AEBP2: AE Binding Protein 2

AMP: Adenosine Monophosphate

AMPK: AMP-Activated Protein Kinase

ATP: Adenosine Triphosphate

CaMK: Ca^{2+}/calmodulin-dependent protein kinase

CAT: Catalase

CC: Capillary Contact

CD: Capillary Density

C:F: Capillary-to-Fiber Ratio

CoA: Coenzyme A

DNMT: DNA Methyltransferases

EC: Excitation-contraction

ERRα: Estrogen-Related Receptor

ETS1: ETS Proto-Oncogene 1

EZH2: Enhancer of Zeste Homolog 2

FOXO1: Forkhead Family Box O1

GH: Growth Hormone

GLUT4: Glucose Transporter Type 4

GPX: Glutathione Peroxidase

H_2O_2: Hydrogen Peroxide

HDAC: Histone Deacetylase

HAT: Histone acetyltransferase

HIF: Hypoxia-Inducible Factor

HMG: High-Mobility-Group

KDR2: Kinase Insert Domain Receptor 2

MAPK: Mitogen-Activated Protein Kinase

Mdm2: Murine Double Minute

MEF: Myocyte Enhancer Factor

MHC: Myosin Heavy Chain

MRF: Myogenic Regulatory Factors

mTOR: Mammalian Target of Rapamycin

MVU: Microvascular Unit

MYOD: Myoblast Differentiation Protein

MYH7: Myosin Heavy Chain 7

NADH: Ubiquinone Oxidoreductase Subunit C2 (NDUFC2)

NFAT: Nuclear Factor of Activated T-cell

NO: Nitric Oxide

PAD: Peripheral Artery Disease

PGC-1α: Peroxisome Proliferator Activated Receptor Gamma Coactivator 1 alpha

PKC: Protein Kinase C (PKC)

PRC: Polycomb Recessive Complex

ROS: Reactive Oxygen Species

SOD: Superoxide Dismutase

TCA: Tricarboxylic Acid Cycle

TGF-beta: Transforming Growth Factor Beta

THBS1: Thrombospondin-1

VEGF: Vascular Endothelial Growth Factor

Chapter 1: Literature Review

This literature review aims to provide the reader with background information that will help in understanding the rationale and results of this thesis. This review will provide in-depth information about skeletal muscle function and plasticity, exercise physiology, adaptations in the skeletal muscle, and epigenetic regulation, primarily focusing on histone modifications.

Skeletal Muscle and its Functions

Skeletal muscle accounts for approximately 40% of human total body mass. It is one of the most dynamic and plastic tissues in our body. The main functions of skeletal muscle are to generate power to produce movement by contraction and maintain posture (Frontera and Ochala, 2015). Skeletal muscle health is crucial to our wellbeing, as it allows for activity and is essential for maintaining independence and supporting whole body metabolism.

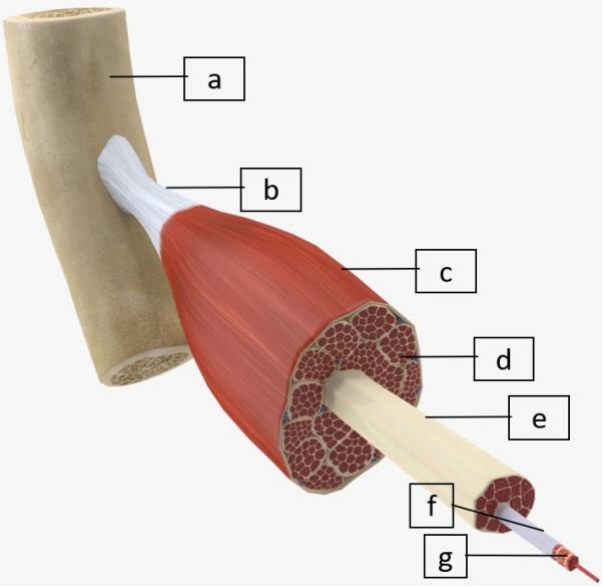


Figure 1.1: Macro-anatomy of the skeletal muscle. This shows a schematic representation of the macrostructure of skeletal muscle. a) bone, b) tendon, c) epimysium, d) endomysium, e) fascicle, f) muscle fiber, g) myofibril.

Skeletal muscles are mainly composed of muscle fibers, also known as myofibers, and connective tissue. Myofibers are arranged into bundles called myofibrils, which are surrounded by perimysium, a type of connective tissue (Fig. 1.1). Myofibrils collectively are the main cellular component of the muscle, which is surrounded by connective tissue called the epimysium (Frontera and Ochala, 2015). The functions of the myofibrils are supported by a network of small blood vessels: the microvasculature. Indeed, skeletal muscle is a highly vascularized tissue. The microvasculature is essential to the function of muscle, as it delivers oxygen and nutrients to the working muscle, and is able to remove metabolic waste.

Satellite and endothelial cells are two important cellular components of skeletal muscle that serve important roles in several physiological processes. Satellite cells, also called myogenic stem cells, are located in the basal lamina of muscle fibers (Mauro, 1961). They are considered the main source of myonuclei in postnatal skeletal muscle (Moss and Leblond, 1970). Satellite cells are usually in a quiescent state in adult skeletal muscle. Upon stimulation, they become activated and are able to proliferate and/or differentiate into myoblasts (Schmalbruch, 1976). Several stimuli, notably exercise, can stimulate satellite cells (Snijders et al., 2015).

Endothelial cells form the lining of blood vessels. Endothelial cells play an important role in regulating blood supply to skeletal muscle. Shear stress, the mechanical forces generated by the friction of blood flow on blood vessel walls, is sensed by endothelial cells through mechanoreceptors. In response to shear stress, endothelial cells initiate signaling cascades to surrounding cells, thereby causing the blood vessel to adjust its diameter to match blood flow (Green et al., 2017). These cells are impacted during skeletal muscle adaptations, as they are able to proliferate and migrate to grow capillaries from pre-existing ones in a process called angiogenesis, which will be discussed in further sections (Hudlicka et al., 1992).

Skeletal Muscle Energetics

The skeletal muscle must undergo contractions in order to produce movement. Muscle contraction is a process that requires high rates of cellular metabolism and energy production. The source of energy utilized during muscle contraction is adenosine triphosphate (ATP). Skeletal muscle contains a small intracellular store of ATP (~5-6 mM). This small store of ATP would be depleted rapidly upon activation of the muscle (Sahlin et al., 1998). Therefore, muscles require other metabolic pathways in order to maintain sufficient ATP. Anaerobic and aerobic metabolism are two major pathways that can be utilized to generate ATP. The anaerobic pathway is fast-acting and short-lived, and is primarily activated at the onset of exercise and during high-intensity, low duration exercise. On the other hand, the aerobic pathway acts slower but has higher longevity, therefore, it is utilized during low-intensity, high-duration exercise (Sahlin et al., 1998). ATP can be generated from several sources: carbohydrates - the primary source - via glycolysis, fats via fatty acid oxidation, and proteins via proteolysis. Metabolism of any of these sources leads to the production of pyruvate and/or acetyl coenzyme A (CoA). When oxygen is present, these new products undergo the tricarboxylic acid (TCA) cycle, followed by the electron transport chain in the mitochondria. These processes generate the highest levels of ATP (Lunt and Vander Heiden, 2011).

Force Production and Contraction by Skeletal Muscle

In order for a muscle to generate force and contract, several preliminary steps must occur. Excitation-contraction (EC) coupling is a coordinated process that allows a muscle to generate force. An action potential from the nervous system arrives at the muscle fiber membrane and is conducted into the fiber via the transverse tubule (T tubule). The impulse arrives in close proximity

to the terminal cisternae of the sarcoplasmic reticulum - the site of calcium storage. Voltage-sensitive receptors on the T tubule open to take in calcium (Rebbeck et al., 2014). The influx of calcium prompts the opening of ryanodine receptors located in the sarcoplasmic reticulum. Upon opening, large amounts of calcium are released into the sarcoplasm, which bind to troponin C on the actin filament (Rebbeck et al., 2014). This causes troponin to shift the position of tropomyosin away from the myosin-binding sites on actin (Lehman et al., 1994). If sufficient ATP is present, myosin is able to bind to actin and proceed to cross-bridge cycling. The sarcomere shortens and the muscle is able to contract (Lehman et al., 1994). Calcium and ATP are crucial cofactors to muscle contraction.

Skeletal Muscle and Glycemic Control

Skeletal muscle plays a major role in substrate oxidation and substrate storage (Egan and Zierath, 2013). Therefore, skeletal muscle highly impacts systemic metabolism including basal metabolic rate, lipid utilization, and insulin-stimulated glucose uptake. Skeletal muscle is responsible for approximately 30% of resting metabolic rate (Zurlo et al., 1990). Skeletal muscle stores the largest amount of glycogen in the body, approximately 4-fold more than the capacity of the liver (Mikines et al., 1988). Exercising skeletal muscle is crucial in glycemic control and metabolic homeostasis, as it is the major site for glucose uptake (Sylow et al., 2017). Indeed, during exercise, glucose-uptake increases considerably. In humans, repeated leg extensions for one hour leads to the increase of muscle glycogen synthase activity and glucose transport, as well as an enhancement in the metabolic action of insulin during the post-exercise period (Richter et al., 1989).

Microanatomy of the skeletal muscle

The skeletal muscle is a highly organized tissue. The microscopic organization of the skeletal muscle plays an integral role in supporting muscle functions. Each muscle fiber has blood supply coming in through nearby capillaries. Satellite cells lie along the muscle fiber alongside capillaries (Fig. 1.2). The satellite cells remain in close proximity to capillaries in every state, whether quiescence, proliferation, or differentiation (Christov et al., 2007). Muscles are composed of a mass network of microvascular units (MVUs). Endomysial capillaries run parallel to muscle fibers. The capillaries which are supplied by the terminal arteriole direct blood to be collected by the venule. The space between the terminal arteriole and the venule represents one microvascular unit. Microvascular units consist of 5-10 capillaries among 3-4 muscle fibers (Latroche et al., 2015).

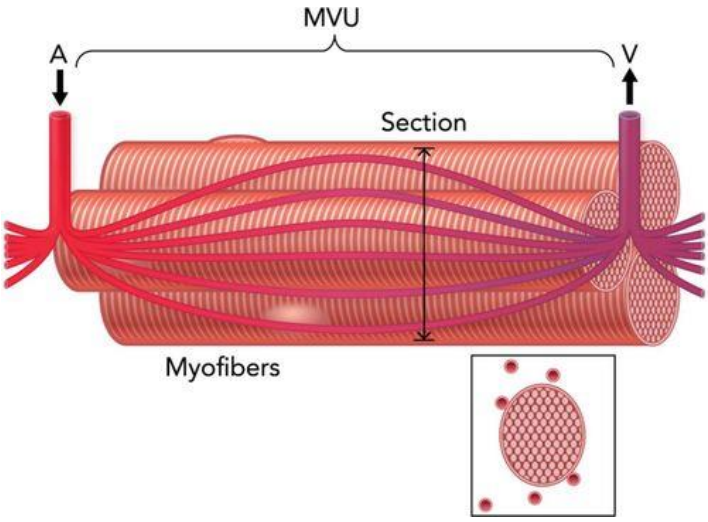


Figure 1.2: Microanatomy of the skeletal muscle. The main panel shows a schematic representation of the three muscle fibers and their microvascular unit (MVU). The right bottom panel represents the spatial relationship of the MVU with a muscle fiber. (Figure adapted from (Latroche et al., 2015).

Muscle Fibers

There is a great degree of heterogeneity among skeletal muscles. Different muscles have different relative compositions of the three major myofiber types. This diversity represents different patterns of movement, thus different adaptations in each muscle type (Frontera and Ochala, 2015). This variation in fiber types is important, as it allows skeletal muscle to meet a wide variety of metabolic and mechanical demands. Classification of muscle fibers is typically based on myoglobin content, contractile properties, speed of contraction and ability to handle calcium, fatigability, and the predominance of metabolic or enzymatic pathways among others (Frontera and Ochala, 2015).

One of the key descriptors of muscle fibers is the speed of contraction, or in other words, whether the fiber is slow-twitch or fast-twitch. Slow-twitch fibers, also called type I fibers, rely on oxidative metabolism as their energy source. These fibers are best suited to long duration, low intensity exercise. Slow-twitch fibers have a higher capillary density compared to fast-twitch fibers (Sjøgaard, 1982). This allows for sufficient oxygen delivery, thus a high resistance to fatigue. Additionally, slow-twitch fibers have slow contractile activity and more mitochondria than other fiber types, allowing them to rely on aerobic respiration (Rasbach et al., 2010). The Myosin Heavy Chain 7 (MYH7) gene codes for the protein beta-myosin heavy chain, which is found in the heart and in type I skeletal muscle fibers. MYH7 is highly associated with slow-twitch fibers in humans (Talbot and Maves, 2016). An example of a predominantly slow-twitch fiber muscle is the soleus in the lower limbs.

In humans, fast-twitch fibers can be classified as type IIA or type IIX. These fiber types have fast contractile activity, but are less resistant to fatigue. Fast-twitch fibers are best suited for rapid bursts of activity. Type IIX fibers rely on glycolytic metabolism as their energy source,

whereas type IIA fibers rely on oxidative metabolism (Talbot and Maves, 2016). Type IIX fibers have low amounts of mitochondria, and low capillarization, however type IIA fibers have a similar oxidative profile to type I with higher levels of mitochondria and capillarization (Lee et al., 2017). Type IIA fibers are considered an intermediate fiber between type I and type IIX, as they display the oxidative profile of type I fibers, however they are fast in terms of force generation/contractile activity similar to type IIX fibers (Qaisar et al., 2016).

The Role of Microvasculature in the Skeletal Muscle

Skeletal muscle is a highly vascularized tissue. Vascularization is an important feature of skeletal muscle, due to the demand for adaptation which considerably depends on the microvasculature. These blood vessels also play a role in skeletal muscle homeostasis (Latroche et al., 2015). Microvasculature plays a role in meeting demands during muscle contraction, which is highly dependent on the metabolism of energy substrates such as glucose.

The heterogeneity among skeletal muscles is also characterized by the capillarization of different muscles. Muscles that predominantly rely on oxidative metabolism will have higher capillarization than muscles that rely on glycolytic metabolism (Sjøgaard, 1982). Capillaries are the smallest blood vessels, and are the primary site for oxygen exchange from the blood to the muscle (Lo et al., 2003). The anatomy of a capillary is a single layer of endothelial cells surrounded by a basement membrane containing pericytes. This anatomical structure makes capillaries an effective site for gas exchange (Hwa and Aird, 2007). Pericytes are essential for endothelial cell survival and the maintenance of the capillary barrier function. In addition, pericytes facilitate blood flow regulation and immunomodulation (Armulik et al., 2011; Bergers and Song, 2005; Navarro et al., 2016). Capillarization is essential for the perfusion of muscle, allowing for sufficient oxygen supply. Capillarization of a muscle can be measured by several indexes, including capillary density

(CD), capillary-to-fiber ratio (C:F), and capillary contact to fiber (CC) (Harris, 2005; Qu et al., 1997). Capillary density can be defined as the number of capillaries per unit cross-sectional area of muscle. Capillary-to-fiber ratio measures the number of capillary profiles per number of fiber profiles. Capillary contact to fiber measures the number of capillaries in contact with an individual fiber (Harris, 2005). The level of capillarization in a tissue depends on the metabolic demands of that tissue (Adair et al., 1990).

The vascular, metabolic, and fiber type profiles of lower limb muscles

Soleus

The soleus muscle is a postural muscle on the calf that contains ~84% type I slow-twitch fibers, and ~16% type IIA fast-twitch oxidative fibers in humans, whereas the mouse soleus shows ~50% type I fibers (Timson et al., 1985; Trappe et al., 2001). Type I fibers have a higher abundance of mitochondria compared to type II fibers. Therefore, type I fibers rely more on oxidative metabolism compared to the other fiber types. The soleus has higher levels of mitochondrial biogenesis to support its oxidative phenotype, compared to fast-twitch muscles (Crupi et al., 2018). Predominantly slow-twitch muscles such as the soleus are better able to maintain mitochondrial homeostasis, and are better protected from mitochondrial functional decline and autophagy as age increases (Crupi et al., 2018). Exercise induces higher levels of oxidative metabolism, which is a driving factor for mitochondrial biogenesis and maintenance of mitochondrial function. In addition, mitochondrial health is causally related to maintaining muscle mass. Short et al. have shown that 16 weeks of stationary bike aerobic exercise effectively slows down age-related mitochondrial degeneration in humans (Short et al., 2003). The soleus shows a significantly higher number of capillaries per fiber, compared to fast-twitch muscles such as the triceps brachii

(Sjøgaard, 1982). In addition, slow-twitch fibers show more capillaries around each fiber compared to fast-twitch fibers (Sjøgaard, 1982).

Interestingly, compared to fast-twitch fibers, slow-twitch fibers are more efficient at scavenging H_2O_2, as they show higher levels of activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) (Criswell et al., 1993; Lawler et al., 1994; Powers et al., 1994). Given that the soleus has predominantly oxidative properties in both humans and mice, it follows that the soleus is a good model to study characteristics of slow-twitch oxidative fibers.

Gastrocnemius

The human gastrocnemius muscle sits over the soleus and has two heads: medial and lateral. The gastrocnemius is a mixed fiber-type muscle. In humans, it contains ~57% type I slow-twitch fibers, 31% type IIA fast-twitch oxidative fibers, and ~11% fast-twitch glycolytic fibers (Trappe et al., 2001). In mice, however, there is a much higher percentage of type II fibers, accounting for 90-95% of total fiber population. More specifically, mice show ~30% type IIA, 50% type IIB, and 10-15% type IIX (Ringseis et al., 2020). In humans, capillarity in the gastrocnemius is very heterogeneous, due to the mixed-fiber phenotype (Brasseur et al., 1987).

An increase in contractile activity and physical activity trigger several changes in the cellular environment, such as calcium cycling, ROS production, oxygen consumption, among others, which result in activation of kinases and subsequent post-translational modifications (Sakamoto and Goodyear, 2002). Most notably, exercise activates the AMP-activated protein kinase (AMPK), among other kinases which are related to mitochondrial biogenesis (Ljubicic and Hood, 2008). Jensen and colleagues later found that kinase activation by 45 minute inclined walking exercise at 69% VO_2 max is higher in the gastrocnemius than in the soleus in humans

(Jensen et al., 2012). This could be due to the fiber-type composition of the gastrocnemius, as it shows a more robust adaptation towards the oxidative phenotype than the soleus, which is already predominantly composed of type I fibers. Additionally, the differences in kinase activation could also be due to the different functional roles of the gastrocnemius compared to the soleus. The soleus muscle is postural, therefore undergoes regular contractile activity. The gastrocnemius undergoes a greater increase in contractile activity than the soleus in response to physical activity, especially during running exercise.

The gastrocnemius fast-twitch fibers also show higher levels of H_2O_2 release compared to slow-twitch fibers. This occurs because the gastrocnemius has more fast-twitch fibers, which are not as well equipped to scavenge ROS. Fast-twitch fibers show significantly lower levels of antioxidant enzymes compared to slow-twitch fibers (Criswell et al., 1993; Lawler et al., 1994; Powers et al., 1994). This characteristic partially explains why the gastrocnemius is more prone to fatigue.

Plantaris

The plantaris is a small muscle on the back of the lower leg in humans and mice. It is a predominantly fast-twitch muscle, as it has ~86% fast-twitch fibers in mice (~72% type IIA fast-twitch oxidative, and ~14% type IIX fast-twitch glycolytic fibers) (Ishihara and Taguchi, 1991). The plantaris plays a major role in proprioception, as it has a high density of muscle spindles (Vlaic et al., 2019).

The plantaris is a highly adaptable muscle. Indeed, wheel-running exercise induces a 3-fold increase in slow myosin isoenzymes, indicating the initiation of fiber-type switching to type I fibers in rats (Gregory et al., 1986). Another study showed that eight weeks of wheel-running exercise training in rats was adequate to change the MHC composition in the plantaris where type

IIB fibers decreased and type I fibers showed a concomitant increase (Fuller et al., 2006). 3 to 4 weeks of treadmill running in rats was also shown to increase the diameter of arterioles in the plantaris, which may be an important step in the adaptation to flow-mediated dilation (Sun et al., 1998).

Plasticity of the muscle phenotype in response to endurance training

Skeletal muscle has the ability to adapt to many different stimuli due its high degree of plasticity. Behavioural factors, such as physical activity, have the capacity to change the microenvironment in the skeletal muscle. This can lead to changes in muscle metabolism, microvascular remodelling, and distribution of fiber type. These changes include mitochondrial biogenesis, angiogenesis, and fiber-type switching. Some of the key stressors that can change muscle phenotype include contractile activity, mechanical and shear stress, metabolic cues, and growth factors (Flück and Hoppeler, 2003). Adaptations occur through changes in the metabolic and contractile profiles of the muscle. Changes in fiber type, metabolic profile, and capillarization are examples of major adaptations that occur in response to these stimuli (Flück and Hoppeler, 2003). These adaptations allow skeletal muscle to meet the energetic and mechanical demands of increased contractile activity, such as during exercise (McGee and Walder, 2017). During exercise, skeletal muscle is able to augment its ability to deliver substrates and oxygen, thereby increasing energy production through aerobic metabolism (Clausen and Trap-Jensen, 1970).

What happens during exercise?

At the onset of exercise, muscle cells use the small amount of ATP stored. The use of this ATP does not rely on oxygen, however, this amount is enough only to last a few seconds into

exercise. Depending on the demands of exercise, cells synthesize ATP through either anaerobic or oxidative metabolism. During endurance exercise, aerobic metabolism is preferred, as this system is better suited for long duration exercise and synthesizes the most ATP (Egan and Zierath, 2013). The energy imbalance caused by exercise leads to an increase in intracellular AMP and ADP concentration, which stimulates AMPK activation (Kjøbsted et al., 2018). The AMPK pathway acts rapidly to bring energy levels back to homeostasis. Working muscles require more blood flow due to higher demand for oxygen and nutrients, thus cardiac output, blood pressure, and vascular resistance in other areas of the body increase.

To initiate muscle contraction, muscle cells are activated at the L-type calcium channels by depolarization. This action triggers the opening of ryanodine receptors and leads to an increase in intracellular calcium, which then activates contractile proteins in muscle cells (Kuo and Ehrlich, 2015). Oscillations in calcium levels during exercise stimulate calmodulin signaling, which will eventually lead to fiber-type adaptations, with repeated bouts of exercise (Chin, 2010). This will be discussed in greater detail in other sections.

Shear stress is a hemodynamic stimulus that is detected by mechanoreceptors in endothelial cells. This mechanical stimulus is converted to chemical signals that initiate intracellular signaling cascades. These signaling pathways can then impact transcription factors for genes that regulate endothelial cells (Green et al., 2017). The altered gene expression can eventually lead to adaptations such as angiogenesis with repeated bouts of exercise. For example, shear stress causes an increase in the production of nitric oxide (NO) in endothelial cells, which is a key signaling molecule for angiogenesis, among other physiological functions (Ziche and Morbidelli, 2000). Interestingly, shear stress-driven NO has the capacity to increase VEGF-A production in endothelial cells, and potentially in myocytes (Egginton, 2011; Uchida et al., 2015). Shear stress

is also able to activate pathways such as the mitogen-activated protein kinase (MAPK) pathway. MAPKs regulate the phosphorylation of a wide variety of substrates involved in differentiation, hypertrophy, inflammation, and gene expression (Long et al., 2004). During contraction, p38 MAPK can stimulate transcription factors of genes that allow the muscle to rely more on oxidative metabolism, such as PGC-1α, and MEF2, among others (Akimoto et al., 2005). Interestingly, shear stress signaling can mediate angiogenesis by activating p38 MAPK and the VEGF receptor 2 (Gee et al., 2010).

Resistance and endurance training stimulates the secretion of growth hormone (GH) from the pituitary gland in humans. Approximately 15 minutes after the initiation exercise, plasma levels of GH increase. GH is a potent anabolic agent, which may play a role in exercise-induced skeletal muscle hypertrophy (Godfrey et al., 2003). GH has also been shown to acutely stimulate muscle protein synthesis in adults (Fryburg et al., 1991). In addition, hypoxia-inducible factor (HIF) and growth factors such as VEGF are impacted during exercise. HIF is a transcription factor composed of HIF-1α and HIF-1β. HIFs play a role in hypoxia-induced VEGF gene expression under a range of oxygen tensions, including those which occur during exercise (Gassmann and Wenger, 1997). In humans, VEGF expression is significantly increased during one bout of cycling exercise due to the increased metabolic demand. Both HIF-1α and HIF-1β showed increased expression alongside the increase in VEGF. However, only HIF-1β showed a significant increase in expression (Gustafsson et al., 1999).

Taken together, these data show the vast amount of changes that occur from the tissues down to cellular levels. Many of these acute changes result in adaptations upon training.

Mechanisms controlling change in fiber type

Regular repetition of bouts of exercise during training can cause changes in fiber type proportions. Endurance training in particular induces a shift toward a higher proportion of type I fibers. Endurance training effectively leads to a transition from fast-twitch fibers to slow-twitch fibers (Lee et al., 2017). Several factors regulate the transition from fast-twitch to slow-twitch fibers. There are two main factors that regulate the conversion between fiber types: MyoD and myogenin activity, and NFAT activity (Zhu et al., 2013; Ehlers et al., 2014).

The expression of the transcription factor MyoD is an essential regulator of myogenesis and is involved in processes that determine muscle plasticity (Tapscott, 2005). MyoD expression changes in a sensitive manner in response to increased or reduced mechanical stress, as well as to changes in electrical activity (Legerlotz and Smith, 2008). MyoD is part of the family of myogenic regulatory factors (MRFs), which also include myogenin, Myf5, and Myf6 (Sabourin and Rudnicki, 2000). Both MyoD and myogenin have been found to play a role in the determination of muscle fiber type during development, as well as the conversion between fiber types. MyoD is highly expressed in fast-twitch glycolytic muscles, whereas myogenin shows higher expression in slow-twitch oxidative muscles, such as the soleus (Ekmark et al., 2007; Hughes et al., 1993, 1997). Interestingly, endurance training led to the increase in mRNA levels of myogenin and oxidative enzymes, however, no effect was seen in the expression of MyoD in rat soleus muscle (Siu et al., 2004).

The nuclear factor of activated T-cell (NFATs) are a group of five transcription factors: NFATc1-4, and NFAT5. These transcription factors are regulated by calcineurin (Hogan et al., 2003). Calcineurin is a phosphatase that is involved in intracellular signaling to maintain the slow-twitch fiber phenotype in skeletal muscle (Hogan et al., 2003). NFAT proteins play a major role

in regulating gene expression of slow-twitch fiber genes, as seen in C2C12 cells (Chin et al., 1998; Wu et al., 2000). Low-amplitude calcium waves initiate the activation of this pathway by the binding of calcium to calmodulin. Calcineurin then acts to dephosphorylate the NFAT which then translocates from the cytoplasm to the nucleus, where it can associate with other transcription factors to activate specific genes that are dependent on calcium. The transcription factor MEF2 can interact with NFAT to activate genes (Rao et al., 1997). In vivo, NFATc1 has been found to be required to modulate the balance of fast- and slow-twitch fiber type conversion in response to 7 days of voluntary exercise in mice (Ehlers et al., 2014). Interestingly, NFATc1 inhibits MyoD function, as well as the expression of MyoD-dependent genes in fast-twitch fibers (Ehlers et al., 2014). Additional evidence to support the role of calcineurin in maintaining the slow-twitch phenotype can be seen in rats treated with cyclosporine A, which is an inhibitor of calcineurin activity. Cyclosporine treatment leads to activation of glycolytic enzymes and a reduction in slow-twitch type I contractile proteins. This promotes a transformation of fibers toward a fast-twitch phenotype (Bigard et al., 2000; Chin et al., 1998). These data show that calcineurin is indeed required for the maintenance of the slow-twitch phenotype.

Pathways controlling the metabolic phenotype

The peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) is a transcriptional cofactor. It is required for the regulation of genes responsible for the metabolic programs in skeletal muscle (Handschin and Spiegelman, 2006). PGC-1α is considered a major regulator of mitochondrial gene expression. It is able to activate mitochondrial biogenesis and oxidative metabolism (Puigserver et al., 1998; Vega et al., 2000; Wu et al., 1999). PGC-1α plays a partial role regulating the response to exercise by skeletal muscle (Olesen et al., 2010). A single bout of voluntary running in mice induces an upregulation in PGC-1α mRNA and protein

expression (Akimoto et al., 2005; Baar et al., 2002; Pilegaard et al., 2003; Terada and Tabata, 2004). This stimulates the conversion of fast-twitch fibers to more slow-twitch oxidative fibers, as well as mitochondrial biogenesis and other exercise-adaptive responses in the muscle (Lin et al., 2002; Wu et al., 1999). PGC-1α plays an important role in orchestrating a broad range of adaptive responses to exercise training by the skeletal muscle.

Microvascular remodelling in response to endurance training

The microvascular bed in skeletal muscle is highly plastic and is able to adapt to several physiological factors including exercise, hypokinesis, hypoxia, and aging. This process can be termed microvascular remodelling and includes angio-adaptation, and potential arteriolar remodelling. This mechanism for adaptation can be termed angio-adaptation (Olfert and Birot, 2011). Adaptations to these physiological changes can occur in the form of capillary growth (angiogenesis), regression or stabilization (quiescence) (Adair et al., 1990). Endurance exercise is a potent stimulus of angiogenesis. This adaptation ensures that the increased metabolic demand is matched by an increased capacity for gas exchange, which is possible with the expansion of the vascular bed.

Angiogenesis

Angiogenesis refers to the process by which new capillaries grow from pre-existing ones. Angiogenesis can be stimulated by physiological conditions such as exercise to meet the increased metabolic demand, and can also be stimulated by pathogenic conditions such as peripheral artery disease (PAD), where there is not enough tissue capillarization (Carmeliet, 2003). In both situations, angiogenesis occurs as a mechanism to maintain homeostasis in the body. Angiogenesis also provides the benefit of shorter diffusion distance (Carrow et al., 1967). In homeostatic

conditions, there is a balance between angiogenic and angiostatic factors. When metabolic demand increases, or in pathological conditions, there is a shift toward pro-angiogenic factors (Olfert and Birot, 2011).

Exercise-Induced Angiogenesis

The vascular system is essential for supplying oxygen and nutrients to tissues, and removing metabolic waste. Initiation of exercise causes an increase in muscular demand for these vascular functions. To meet these demands, heart rate and cardiac output are elevated. In addition, blood is redirected to the exercising muscle decreasing vascular resistance to vessels supplying the muscle, while increasing vascular resistance in other areas (Whyte and Laughlin, 2010). However, these responses alone may not be adequate to meet the increased metabolic demand in untrained muscles. An expansion of the microvascular bed by angiogenesis is required to optimize oxygen and nutrient delivery, and waste removal.

Exercise is a potent angiogenic stimulus. Repeated increases in contractile activity can induce an increase in the number of capillaries in as little as a 4 to 7 days (Egginton, 2009). Vascular endothelial growth factor-A (VEGF-A) has emerged as an important pro-angiogenic factor, as it promotes endothelial cell proliferation, survival, and migration (Carmeliet and Jain, 2011). An acute bout of treadmill running in mice causes an increase in circulating levels of VEGF-A, as well as mRNA levels after cessation of exercise (Gavin and Wagner, 2001; Kraus et al., 2004). Exercise-induced angiogenesis can be stimulated by hypoxia and mechanical stress, such as shear stress. Capillary growth can occur by sprouting or splitting angiogenesis.

Several factors serve as intracellular regulators of angiogenesis. HIFs work under low oxygen conditions. Notably, HIF-1α is able to regulate VEGF-A transcription, as well as increase the production of angiopoietin-2, another pro-angiogenic factor (Mandriota and Pepper, 1998).

Through its interaction with estrogen-related receptor (ERR)α, PGC-1α enhances VEGF transcription in response to hypoxia and under metabolic stress (Arany et al., 2008). The Forkhead family box O1 (FoxO1) is an anti-angiogenic transcription factor that regulates the transcription of several targets including cyclinD1, and thrombospondin-1 (another anti-angiogenic factor) (Paik et al., 2007; Roudier et al., 2013). The tumor-suppressor p53 is an anti-angiogenic factor, as it inhibits the expression pro-angiogenic factors and stimulates the expression of anti-angiogenic factors (Qin et al., 2006).

Murine double minute-2 (Mdm2) is an E3 ubiquitin ligase that negatively regulates p53 (Prives, 1998). Although Mdm2 has primarily been studied in the context of oncology, it has recently emerged as a pro-angiogenic factor, as it is a key regulator in exercise-induced angiogenesis in skeletal muscle. 8 weeks of running exercise training enhances Mdm2 expression in rat skeletal muscle (Roudier et al., 2012). The role of Mdm2 as a pro-angiogenic factor is supported by the knowledge that it promotes HIF-1α stabilization to facilitate VEGF-A transcription (Carroll and Ashcroft, 2008). Additionally, phosphorylation of Mdm2 leads to the reduction of FoxO1 protein expression (Aiken et al., 2016). Interestingly, shear stress has been shown to activate Mdm2 to suppress FoxO1 expression (Milkiewicz et al., 2011).

Exercise-induced angiogenesis occurs in a differential manner specific to fiber types. In the glycolytic plantaris, capillary density and capillary-to-fiber ratio increased twofold after four weeks of voluntary exercise in mice. Following this increase, muscle fibers in the plantaris transformed to a more oxidative phenotype (Waters et al., 2004). Another study that observed 12 to 14 weeks of high intensity endurance running in rats induced changes in the oxidative soleus muscle and the mixed gastrocnemius muscle revealed that angiogenesis occurs in a nonuniform manner among these different fiber types. Gastrocnemius white fibers showed a 58% increase in

capillary-to-fiber ratio, gastrocnemius mixed fibers showed a 38% increase, gastrocnemius red fibers showed a 15% increase and the soleus - predominantly red fibers - showed only a 7% increase (Gute et al., 1996).

Taken together, these data show that microvascular remodelling by angiogenesis is a complex but physiologically beneficial adaptation to exercise.

Modulators of Muscle Phenotype

The plasticity of muscle, as well as fiber type heterogeneity is dependent on muscle phenotype. There are several mechanisms by which skeletal muscle adaptations can occur, including changes to fiber type. Gene expression in skeletal muscles is regulated by a few key pathways: MEF2, calcium signaling, and CaMK signaling.

Pathways controlling change in gene expression during skeletal muscle fiber-type switching

MEF2

The myocyte enhancer factor-2 (MEF2) is a family of transcription factors that play a major role in the formation of muscle fibers by activating muscle-specific genes. MEF2 works alongside histone deacetylase (HDAC) enzymes in response to changes in intracellular calcium, which occur due to physiological stressors, such as exercise. This joint pathway acts to transform myofibers by targeting gene expression (Bassel-Duby and Olson, 2006). MEF2 DNA binding has been seen in response to insulin, H_2O_2, osmotic stress, and activation of AMPK in cell culture models of human skeletal muscle cells (Al-Khalili et al., 2004).

Voluntary wheel-running exercise in mice has been shown to promote MEF2 activity and promote transformation of fiber type, where type IIB fibers are converted to IIA, and type IIA fibers are converted to type I (Wu et al., 2001). In addition, proteins associated with oxidative metabolism, such as myoglobin, were upregulated (Wu et al., 2001). Activation of the MEF2 and calcineurin pathways increase expression of genes including myoglobin, myosin heavy chain, and slow troponin I (Chin et al., 1998). Interestingly, myoglobin and slow troponin I genes are selectively expressed in slow-twitch oxidative muscle fibers (Garry et al., 1996; Levitt et al., 1995). These data reveal that the MEF2 and HDAC pathway also interact with calcineurin signaling to allow myofibers to adapt contractile and metabolic features induced by changing patterns of muscle contraction as a result of exercise.

Calcium/Calmodulin-Dependent Protein Kinase and Protein Kinase C/D

Class II HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) bind to MEF2 and cause repression of MEF2-dependent genes. Class II HDACs are highly expressed in skeletal muscle (McKinsey et al., 2001). Phosphorylation of the class II HDACs causes them to activate MEF2-dependent genes as well as translocate out of the nucleus. Activation of these genes leads to muscle remodelling (McKinsey et al., 2000). Given that phosphorylation plays such a crucial role in gene activation that allows for myocyte differentiation and remodelling, studying kinases that regulate class II HDACs has been of particular interest. Signaling by calcium/calmodulin-dependent protein kinase (CaMK) has been shown to phosphorylate class II HDACs by promoting the translocation of HDACs from the nucleus to the cytoplasm and by the activation of MEF2 (McKinsey et al., 2000).

CaMKII is sensitive to the frequency of calcium oscillations (Koninck and Schulman, 1998). In addition, CaMKII has shown to be activated during hypertrophic growth of the skeletal

muscle, as well as during endurance training-induced adaptations (Chin, 2004). The protein kinase C (PKC) pathway also plays a role in the activation of class II HDACs, as it promotes the phosphorylation of HDAC5 (Vega et al., 2004). Protein kinase D (PKD) acts as a downstream effector of PKC and stimulates the translocation of HDAC5 out of the nucleus (Kim et al., 2008). Taken together, these data reveal the importance of calcium signaling in the gene activation of MEF2-dependent genes that will ultimately aid in adaptations toward the slow-twitch phenotype. They also highlight the central role of HDACs (Fig. 1.3).

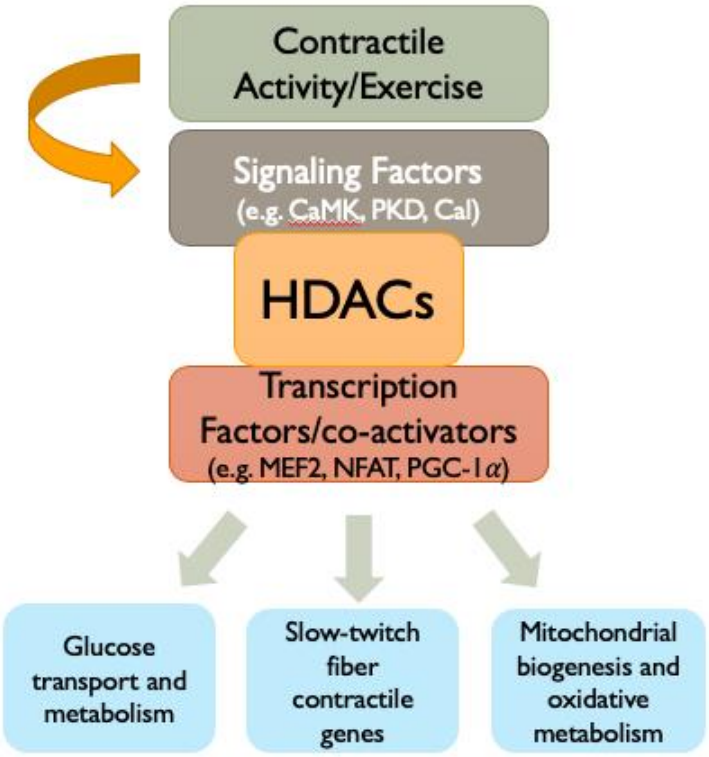


Figure 1.3. Schematic showing the regulation of relevant properties of the oxidative phenotype. At basal levels with regular contractile activity, or with exercise, signaling factors such as calmodulin, protein kinase D, and calcineurin can be stimulated, which will then interact with transcription factors or co-activators to support glucose transport and metabolism, activate genes involved in the slow-twitch fiber type, and activate mitochondrial biogenesis and oxidative metabolism. HDACs are involved in some of these pathways for epigenetic regulation of target genes.

Epigenetic Regulation of Muscle Phenotype

So far, we know that the phenotype and plasticity of skeletal muscle is highly dependent on gene expression. We have seen the importance of HDACs play a role in fiber-type switching (Fig. 1.3), however they also play a major role in the epigenetic regulation of gene expression. HDACs primarily function through direct modifications of histones, which is a type of epigenetic regulation of gene expression. This link has sparked interest in researchers to further investigate the role of epigenetic processes in controlling skeletal muscle phenotype.

What is epigenetics?

Epigenetic regulation is another level of regulation that facilitates skeletal muscle plasticity. Epigenetics is the study of gene expression that occurs in response to environmental stimuli, independent of genotype. More specifically, epigenetics has been defined as "the structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states" (Bird, 2007). DNA methylation and post-translational modifications (PTMs) of histones are two of the most studied topics of epigenetics. These two types of modifications change the chromatin structure to allow for the repression (i.e. silencing) or activation of genes. Other epigenetic modifications can arise from non-coding RNAs, predominantly microRNAs.

MicroRNAs

MicroRNAs (miRNAs) play a role in epigenetics, as they are able to alter gene expression by modulating post-transcriptional activity and translational events in a tissue specific manner. miRNAs modulate several processes such as proliferation, differentiation, and apoptosis by regulating the expression of genes. Altered expression of these genes has been associated with

several human pathological conditions, such as cancer and cardiac failure (Ardekani and Naeini, 2010; Pandey et al., 2009). miRNAs regulate the mRNA degradation and translational repression of other epigenetic modifiers, such as DNA methyltransferases (DNMTs) and HDACs. Interestingly, miRNAs themselves can also be epigenetically regulated (e.g. by DNA methylation and histone modifications) (Poddar et al., 2017).

DNA Methylation

DNA methylation has been the most highly studied topic in epigenetic regulation in the skeletal muscle. It is the process by which a methyl group is added to the 5-carbon position of cytosine bases by the action of DNMTs. Plant and animal studies have both shown that DNA methylation primarily results in gene silencing (Bird and Wolffe, 1999). DNA methylation may also play a role in the etiology of some pathological conditions such as diabetes. Indeed, cytosine hypermethylation of the promoter region of PGC-1α is correlated with reduced mitochondrial content in skeletal muscle of type 2 diabetes patients (Barrès et al., 2009).

Skeletal muscle adaptations to exercise require transient increases in mRNA for genes controlling the metabolism or structure of skeletal muscle. When exercise is repeated chronically during training, these changes in gene expression support skeletal muscle adaptation (Williams et al., 1986). Acute changes to gene expression after a single bout of exercise are different after the robust adaptations of muscles exercise. In humans, a single bout of leg-extension exercise leads to an alteration of the expression of over 1,000 genes in skeletal muscle. After a long term training regimen on a cycle ergometer, however, a bout of leg-extension exercise leads to changes in gene expression of approximately 220 genes, which is much fewer genes compared to untrained skeletal muscle (Popov et al., 2019).

There is a global reduction in DNA methylation after acute exercise on a cycle ergometer in humans, indicating that this change plays a role in the exercise-induced gene expression. Exercise leads to increased expression of genes associated with skeletal muscle adaptations, such as PGC-1α and MEF2. This occurred with a concomitant reduction in DNA methylation. This effect was more pronounced at higher exercise intensities (Barrès et al., 2012). Endurance training has been associated with significant changes in the distribution of DNA methylation in the skeletal muscle (Lindholm et al., 2014). For example, DNA is hypomethylated on specific loci of metabolic genes, such as NADH:ubiquinone oxidoreductase subunit C2 (NDUFC2) and MEF2A (Lindholm et al., 2014; Nitert et al., 2012). Genes associated with angiogenesis such as KDR2, are also hypomethylated after training. The KDR2 gene codes for the VEGF receptor 2, a key regulator of angiogenesis (Lindholm et al., 2014). Kanzleiter et al. revealed that 9 weeks of treadmill running exercise in mice affects several biological pathways in terms of DNA methylation and gene expression. The genes involved in the MAPK, mTOR, Wnt, and TGF-beta signaling pathways are affected by training. The MAPK, Notch, neurotrophin, Wnt, and cancer pathways are some whose gene expression was differentially expressed (Kanzleiter et al., 2015). These data reveal that DNA methylation plays a major role in adaptation of skeletal muscle to exercise and training by regulating gene expression. However, in many cases, changes in DNA methylation are not sufficient to explain the changes in gene expression induced by endurance exercise. Some genes undergo changes by DNA methylation, however, these changes are not always associated with increased gene expression, or vice versa. Indeed, Kanzleiter et al. showed that of the 2659 genes that were differentially regulated, 2401 were differentially methylated, and only 361 genes overlap being both differentially regulated *and* differentially methylated (Kanzleiter et al., 2015). This

illustrates that there are other mechanisms other than DNA methylation for gene expression to occur, such as histone modifications.

Histone Modifications

In the nucleus of the cell, DNA coils around cores of histone proteins. This coiling ultimately results in the formation of chromatin, of which the functional unit is a nucleosome. Histones proteins are grouped together to form a core octamer which consists of two of each type of histones: H2A, H2B, H3, and H4 (Luger et al., 1997). The chromatin structure plays a crucial role in the regulation of gene expression. The modulation of chromatin structure to allow for the silencing or activation of genes can also occur through histone modifications (Fig. 1.4.).

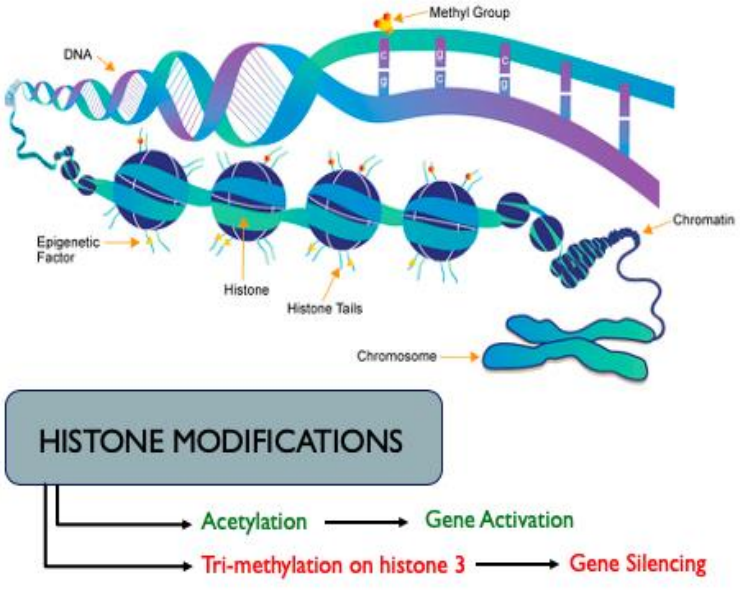


Figure 1.4. Representation of DNA structure from the level of base pairs to chromosomes and some relevant epigenetic modifications. Histone modifications result in the activation or silencing of genes. Acetylation of a histone on a gene typically leads to gene activation. Tri-methylation of histone 3 on a gene typically leads to gene silencing. Figure adapted from epigenetek.com.

Many different post-translational modifications (PTMs) can modify histones to regulate gene expression. These modifications occur primarily on the lysine residues on histone tails, particularly of H3 and H4. Methylation, acetylation, ubiquitination, and phosphorylation are among the PTMs that can modify histones (Li et al., 2007). Modifications can have differential

effects on transcription, depending on the type of modification and the target amino acid residue. For example, methylation of H3 on lysine 4 leads to transcriptional activation, whereas methylation of lysine 36 leads to transcriptional repression (Keogh et al., 2005; Wang et al., 2009). Interestingly, some modifications occur to facilitate the addition of other modifications. For example, monoubiquitination of H3 on lysine 23, 26, and 37 residues is required for inducing acetylation (Zhang et al., 2017). This intermediate step may be of importance to ease the activation of gene expression. The Histone Code aims to characterize all of the potential histone modifications, as well as the interdependencies. Several enzymes, such as HDACs and epigenetic writers, such as enhancer of zeste homolog-2 (EZH2) are involved in the placement of histone modifications. These will be discussed in greater detail below. See table 1.1 for common histone modifications.

The role of histone modifications in the adaptation of skeletal muscle to exercise training has been studied to a much lesser degree compared to DNA methylation. Research thus far has primarily focused on gene activation caused by histone modifications. The role of histone modifications in gene repression, however, has not yet been investigated.

Table 1.1. Common histone modifications and their resulting gene regulation.

Type of modification	**Histone**		
	H3K4	**H3K9**	**H3K27**
Mono-methylation	Activation	Activation	Activation
Tri-methylation	Activation	Repression	Repression
Acetylation	Activation	Activation	Activation

Histone Acetylation, HATs, and HDACs

Histone acetylation is generally associated with transcriptional activation. The acetylation of lysine causes neutralization of the positively charged side chain, effectively disrupting the electrostatic interactions with the negatively charged phosphate backbone of DNA (Li et al., 2007). This opens the chromatin conformation and allows for transcription factors to initiate transcription of these genes. Histone acetyltransferases (HATs) catalyze the acetylation of lysine residues on histones. HATs can be considered transcriptional coactivators due to this characteristic. Histone deacetylases (HDACs), on the other hand, remove these acetyl groups. For this reason, HDACs can be considered transcriptional corepressors (McKinsey et al., 2001). The balance of HAT vs. HDAC activity at a promoter of a gene determines acetylation, or in other words, activation of that gene to undergo transcription. Class II HDACs in particular play a major role in maintaining the slow-twitch phenotype, by associating with MEF2, as previously discussed in the MEF2 section.

HDACs and the AMPK pathway

AMP-activated protein kinase (AMPK) is a heterotrimer that responds to changes in cellular energy levels and cellular stress. These changes are assessed by the AMP-to-ATP ratio. AMPK activation requires phosphorylation by upstream kinases of Thr172. AMPK activity increases during treadmill running exercise in rats (Towler MC and Hardie DG, 2007). This activity is associated with an increase in GLUT4 gene expression. GLUT4 plays an important role in carbohydrate metabolism and the uptake of glucose to the skeletal muscle, including during exercise. Indeed, treatment with an AMPK activator caused increased gene expression of GLUT4 in skeletal muscle through H3 acetylation of the GLUT4 gene (McGee et al., 2008). HDAC5 is considered to be a regulator of the GLUT4 gene. The dissociation of HDAC5 from MEF2, and its translocation out of the nucleus to the cytoplasm is associated with increased GLUT4 gene

expression. In human primary myotubes, differentiation was accompanied by a concomitant increase in muscle-specific myogenic marker expression, including MHC, MyoD, and myogenin. Treatment with an HDAC inhibitor resulted in a 7-fold increase in GLUT4 mRNA, which supports that HDAC plays a role in GLUT4 gene regulation (McGee et al., 2008). The same study also found evidence that AMPK phosphorylation of HDAC5 relieves transcriptional repression of the GLUT4 gene (McGee et al., 2008). Collectively, these data show that cellular energy levels impact gene expression through enzymes involved in histone modifications.

Polycomb Repressive Complex 2

The Polycomb Group is a set of proteins that maintain gene expression patterns of a variety of cells during early development (Margueron and Reinberg, 2011). These proteins act by regulating chromatin structure. There are two main Polycomb groups in mammals: Polycomb repressive complex 1 (PRC1) and 2 (PRC2). PRC1 is involved in catalyzing mono-ubiquitination of H2A and facilitates in compacting chromatin. PRC2 is composed of several components, including enhancer of zeste homolog 2 (EZH2), Jumonji and AT-Rich Interaction Domain Containing 2 (JARID2), and AE Binding Protein 2 (AEBP2), among others. PRC2 is involved in catalyzing the di- and tri-methylation of H3 on lysine 27, through EZH2 which leads to gene silencing (Margueron and Reinberg, 2011). PRC2 also plays a role in chromatin compaction, differentiation, maintaining cell identity and proliferation, and stem-cell plasticity (Shaver et al., 2010). PRC2 can be recruited to genes for transcriptional repression. In Drosophilia, polycomb response elements on the DNA associate with PRC2 and PRC1 (Schuettengruber and Cavalli, 2009). Studies have suggested that the targeting or binding of PRC2 to unmethylated CpG islands may partially be dependent on PRC1 complexes containing CpG island binding proteins (Blackledge et al., 2014). Evidence also shows that PRC2 recruitment is required to establish the

H3K27me3 silencing marks on unmethylated CpG sites after transcription for nucleosome compaction (Jermann et al., 2014). These data may be relevant in explaining PRC2 targeting, however the mechanisms for this remain elusive and require more study, particularly in mammalian cells and animal models. These functions of PRC2 are essential for the maintenance of phenotypic traits such as fiber-type in muscle. Given our interest in gene silencing, more focus will be given to EZH2 compared to the other components of PRC2.

The variety of roles of EZH2 in gene silencing and in skeletal muscle

EZH2 is an epigenetic writer that has been reported to play important roles in the skeletal muscle through the regulation of the regeneration, differentiation and angiogenic capacity of this tissue (Consalvi et al., 2017). The main function of EZH2 is to induce gene silencing by transferring methyl groups to histone 3 at lysine 27 (H3K27me3). Gene silencing by EZH2 is of particular importance in muscle regeneration, where genes must be repressed at different stages of differentiation (Palacios et al., 2010). EZH2 is required for the maintenance of self-renewal and proliferation of satellite cells - a process that is key in muscle regeneration (Juan et al., 2011; Woodhouse et al., 2013). During myoblast differentiation into myotubes, EZH2 levels have been seen to decrease. Once fully differentiated myotubes have formed, EZH2 is barely detectable (Caretti et al., 2004). This suggests that silencing marks may need to be removed for differentiation to proceed.

PRC2 activity can be regulated by several signalling cascades. Interestingly, the p38α MAPK pathway can affect PRC2 function by phosphorylating EZH2 on threonine 372. The phosphorylation causes EZH2 to relocate to the Pax7 promoter. Pax7 is a transcription factor that is involved in myogenesis by regulating satellite cells. Translocation of EZH2 to the Pax7 promoter induces repression of satellite cell differentiation and is essential for cell-cycle exit (Mozzetta et

al., 2011; Palacios et al., 2010). The p38α MAPK pathway has been shown to play an essential role in gene expression regulation during muscle differentiation (Perdiguero et al., 2007; Rampalli et al., 2007; Serra et al., 2007). Consalvi et al. demonstrated that the activation of p38α occurs at the onset of muscle differentiation and this promotes the degradation of EZH2 through phosphorylation of threonine 372 (Consalvi et al., 2017). Interestingly, AMPK has been reported to phosphorylate EZH2 on Thr311. This leads to an inhibition of EZH2 methyltransferase activity (Wan et al., 2018). Since a bout of exercise changes the concentration of intracellular AMP and ADP, which then activates the AMPK pathway, it is plausible that EZH2 activity could be regulated during exercise. However, this remains unexplored.

Mdm2 and its role in skeletal muscle

Mdm2 plays several roles in skeletal muscle cells. Mdm2 is an E3 ubiquitin ligase that has been reported to play a role in maintaining muscle homeostasis. Ramos et al. described that Mdm2 is located in satellite cells in rat skeletal muscle and that activation of Mdm2 supports the maintenance of type 1 fibers (Ramos et al., 2018). We have shown that Mdm2 is required to maintain the level of vascularization of skeletal muscle, even at basal levels in mice, and is required for skeletal muscle angiogenesis in response to endurance training (Roudier et al., 2012). Mdm2 has been shown to play a role in the repression of MyoD, a key regulator of muscle cell differentiation (Fiddler et al., 1996). MyoD is required for maintaining the fast-twitch type IIB/IIX fibers in mice (Hughes et al., 1997). Mdm2 could be key in controlling how muscle fibers acquire one phenotype versus another.

EZH2 and its interaction with Mdm2

A study by Minsky and colleagues revealed that Mdm2 interacts with core histones, and promotes histone ubiquitination in vitro and in vivo. The authors also found that overexpression of Mdm2 enhances protein ubiquitination around the p53 binding site on the promoter. Additionally, Mdm2 was shown to contribute to gene repression through its E3 activity (Minsky and Oren, 2004). This suggests that ubiquitination may also be a mechanism for gene silencing. Wienken et al. found supporting evidence in the role of Mdm2 in gene repression. Interestingly, the researchers revealed that Mdm2 and EZH2 physically associate, and this interaction is important in the resulting gene repression. In fact, Mdm2 was actually found to enhance the trimethylation of H3 (H3K27me3). Indeed, when Mdm2 was removed from MEF cells, several regions of target genes showed a decrease in H3K27me3 (Wienken et al., 2017). Mdm2 was also found to maintain stemness on chromatin by silencing genes involved in cell differentiation through H3K27me3 and H2A119ub1 (Wienken et al., 2016). This ensures that stem cells maintain the capacity to renew themselves and remain in an undifferentiated state. These findings support the idea that Mdm2 could facilitate histone PTMs through its interaction with EZH2 or potentially through direct ubiquitination. These functions of MDM2 and EZH2 interaction remain unstudied in the context of exercise training. A cancer study observing the relationship between EZH2 and Mdm2 in HepG2 and Huh7 cells revealed that EZH2 protein availability improved Mdm2 protein stability (Guo et al., 2018). HBP1 is a transcription factor part of the high-mobility-group (HMG) family. HBP1 is able to repress its target genes at the transcriptional level, including EZH2 (Chen et al., 2016). Interestingly, when Mdm2 was overexpressed in HeLa and H1299 cells, it was able to repress HBP1 expression by reducing its stability through ubiquitination. This promotes genome instability, which is a common characteristic among most human cancers (Cao et al., 2019).

Histone modifications, exercise, and gene expression

Exercise is a potent stimulus for epigenetic changes. Gene expression can be altered by chromatin remodelling through dynamic histone modifications. In human skeletal muscle, a single bout of cycling for 60 minutes has been shown to cause a global increase in H3 lysine 36 acetylation, which is associated with transcriptional activation (McGee et al., 2009). This is one of the many examples of H3 acetylations induced by exercise. Interestingly, the acute bout of cycling for 60 minutes was also shown to alter class IIA HDAC function. Class II HDACs play a major role in skeletal muscle adaptations to exercise training. Immediately after exercise, multiple class IIA HDACs were activated by phosphorylation, and HDAC5 ubiquitination showed a 30% increase (McGee et al., 2009). This suggests that ubiquitin-mediated proteasomal degradation may play a role in the post-exercise period. Indeed, exercise induced HDAC5 translocation from the nucleus to the cytoplasm in human skeletal muscle. The exercise-induced reduction in HDACs decreases the capacity for MEF genes to be deacetylated. Maintenance of acetylation prolongs the opening of the chromatin allowing for the activation of MEF genes. This activity is essential for HDAC interaction with MEF2-dependent genes that will allow for skeletal muscle adaptations to training.

Lochmann et al. showed the importance of histone methylation in the process of exercise adaptation. One bout of rotarod exercise in mice leads to the trimethylation of H3 on lysine 4 (H3K4me3) on the promoter regions of PGC-1α genes. This modification allows for transcriptional activation of the PGC-1α genes (Lochmann et al., 2015). Recall that PGC-1α is a transcriptional cofactor, which is required for the regulation of genes responsible for the metabolic programs in skeletal muscle (Handschin and Spiegelman, 2006). However, conflicting data from Willkomm et al. shows that metabolically demanding high-intensity leg-extension exercise leads

to the reduction of H3K4me3 (Willkomm et al., 2017). The authors suggest that this decrease may be the result of local lactate accumulation. The physiological function of H3K4me3 remains elusive, however it is known that this modification promotes the expression of muscle-specific genes. In addition, modifications of H4 were found to be relevant to the adaptation to exercise. Trimethylation of H4 on lysine 20 (H4K20me3) was induced by exercise training in rats. Oshawa et al. have described that different types of run training in rats leads to the differential regulation of activation marks and the silencing mark H4K20me3 (Ohsawa et al., 2018). These results should be taken with caution, as there were no observations of muscle adaptations in the rats

Interestingly, histone modifications show different patterns in different muscle fiber types. Masuzawa et al. showed that H3 acetylations controlling PGC-1α genes in response to 20 minutes of treadmill running exercise in rats showed profound differences between fast- and slow-twitch muscle types. The soleus muscle, which is predominantly composed of slow-twitch fibers, showed enhanced acetylation of H3 at lysine 27 compared to the plantaris, which is predominantly composed of fast-twitch fibers (Masuzawa et al., 2018). Hindlimb suspension of rats induced fiber-type switching. This occurs as H3 in type I genes become de-acetylated in correspondence to the down-regulation of these genes. Meanwhile, upregulation of the genes involved in maintaining the fast-twitch fiber type occurred, with the support of enhanced H3 acetylation of these genes. Another activation mark, H3K4me3, was also enhanced for genes associated with the fast-twitch phenotype (Pandorf et al., 2009). These data suggest that histone modifications play a role in fiber-type switching, which could also occur in response to exercise.

These data are valuable in describing the changes in gene expression as a result of transcriptional activation modifications. However, we do not yet know the role of silencing marks in the context of skeletal muscle phenotype.

Gene silencing, skeletal muscle phenotype and response to exercise.

Previous literature has shown that an acute bout of exercise induces changes to over 1,000 genes. However, after undergoing exercise training on a cycle ergometer, a bout of exercise impacts far less genes than in untrained muscles (Popov et al., 2019). This suggests that many of those genes are no longer required and are therefore silenced. There is more information about gene silencing in the context of DNA methylation, however, studies are scarce regarding gene silencing in the context of histone modifications. As Lindholm et al. demonstrated, DNA methylation only explains a proportion of changes in gene expression (Lindholm et al., 2014). This provides further rationale to study histone modifications in differential gene expression, as well as in the context of exercise. The maintenance and the plasticity of the skeletal muscle cannot only depend on epigenetic histone activation marks. There is some evidence that indeed suggests that gene silencing might be required from the determination of the skeletal muscle phenotype and the muscle plasticity in response to endurance training.

Slow- versus fast-twitch muscle fibers show phenotypically different characteristics. In addition to factors such as PGC-1α and MEF2 among others, the different phenotypes are maintained in part by epigenetic regulation, including histone modifications. For example, acetylation of histones on MyoD-binding sites in skeletal muscle cells occurs during embryonic myogenesis. This allows skeletal muscle-specific gene regulation by MyoD (Lassar et al., 1991; Tapscott, 2005). The importance of histone modifications in this process suggests that histone modifications also play a role in mediating cell differentiation. Activating histone modifications such as H3K4me3 and H3 acetylation are altered based on the up- or downregulation of the MHC genes for fast-twitch versus slow-twitch muscles, such as the plantaris and soleus, respectively (Pandorf et al., 2009). Kawano et al. compared the activating marks of genes involved in

maintaining the fast-twitch versus slow-twitch phenotype in both the plantaris and soleus muscles. The authors found that the TSS of genes associated with the fast-twitch phenotype showed higher levels of activating histone modifications, than that of genes associated with the slow-twitch phenotype in the plantaris. In the soleus, however, there was no relationship between gene expression and activating histone modifications (Kawano et al., 2015). These differences suggest that the genes involved in maintaining the fast-twitch phenotype may require activating marks to allow for significant gene upregulation. The data may also suggest that genes associated with the slow-twitch phenotype do not require a high degree of upregulation to be expressed, such as in the soleus muscle. Additionally, these data calls into question whether fast-twitch associated genes may actually require silencing in muscles such as the soleus, where slow-twitch associated genes are dominant. These data leave us questioning whether silencing marks are required on fast-twitch muscles, such as the plantaris.

Chapter 2: Study

Introduction

Epigenetic regulation plays an essential role in the maintenance of muscle phenotype. Epigenetics is the study of gene regulation that occurs in response to environmental changes and does not affect DNA sequence. DNA methylation, histone modification, and miRNA regulation are the most common mechanisms of epigenetic regulation. Post-translational modifications (PTMs) by DNA methylation and histone modification induce changes to the chromatin structure to allow for the repression (i.e. silencing) or activation of genes (Bird, 2007). DNA methylation has been studied extensively, both in the context of muscle phenotype and exercise adaptations. Similarly, gene activating histone modifications, such as histone acetylation, have received much more attention compared to gene silencing modifications. Muscle phenotype and gene silencing remain largely unstudied.

Murine double minute-2 (Mdm2) is an E3 ubiquitin ligase that negatively regulates p53 (Prives, 1998). However, Mdm2 has recently emerged as an important pro-angiogenic factor, as it plays a major regulatory role in exercise-induced angiogenesis in skeletal muscle. Our previous work has shown that 8 weeks of treadmill running exercise in rats enhances Mdm2 expression in skeletal muscle and is correlated with an increase in capillary-to-fiber ratio (Roudier et al., 2012).

There is some evidence to suggest that Mdm2 interactions are important in the context of gene silencing. Mdm2 interacts with core histones, and promotes histone ubiquitination. Histone ubiquitination around the p53 promoter is enhanced when Mdm2 is overexpressed. Additionally, Mdm2 contributes to gene silencing through its E3 ubiquitin ligase activity (Minsky and Oren, 2004). These data suggest that ubiquitination may be an important PTM for gene silencing. In a study by Wienken et al., Mdm2 was found to physically associate with the enhancer of zeste

homolog 2 (EZH2). This protein is an epigenetic writer and a major component of the polycomb recessive complex 2 (PRC2) complex. EZH2 is a histone methyltransferase, which places three methyl groups onto histone 3 at lysine 27 (H3K27me3) to induce gene silencing. During myogenesis, EZH2 levels are depleted, as seen in C2C12 cells (Caretti et al., 2004). This suggests that silencing marks may need to be removed for differentiation to proceed, however, the mechanism for this remains elusive. The interaction between Mdm2 and EZH2 was found to enhance the trimethylation of H3 at lysine 27 (H3K27me3). Indeed, when Mdm2 was knocked down in MEF cells, several regions of target genes showed a decrease in H3K27me3 (Wienken et al., 2017). These data provide valuable information about the role of Mdm2 and EZH2 interactions in gene silencing. However, the role of Mdm2-EZH2 interaction has not been investigated in the skeletal muscle or in the context of muscle remodelling.

Slow- versus fast-twitch muscle fibers show phenotypically different characteristics. Activating histone modifications such as H3K4me3 and H3 acetylation are altered based on the up- or downregulation of the MHC genes depending on the muscle phenotype (Pandorf et al., 2009). Kawano et al. compared activation marks of genes involved in the control of contractile and/or metabolic adaptation in fast-twitch versus slow-twitch fibers in the plantaris (a predominantly fast-twitch muscle) and the soleus (a predominantly slow-twitch muscle). They found that genes involved in regulating the properties of fast-twitch fibers showed higher levels of activating histone modifications, than that of genes involved in regulating slow-twitch fibers in the plantaris on the transcriptional start site (TSS). In the soleus, however, there was no relationship between gene expression and activating histone modifications (Kawano et al., 2015). The difference in the regulation of genes involved in controlling fast-twitch fiber properties and that of slow-twitch fibers suggests that genes controlling fast-twitch properties require activating marks

to allow for significant gene upregulation. This also suggests that genes involved in controlling the slow-twitch phenotype do not require significant activation. This data asks the question of what the role of silencing marks may be in the regulation of fast-twitch genes. Are fast-twitch genes silenced in the predominantly slow-twitch muscles? To date, no study has answered this question.

Gap of Knowledge

In order for myogenesis or muscle adaptations to occur, gene regulation is necessary to ensure that appropriate genes are activated or repressed to produce that particular phenotype. Presently, there remains a major gap of knowledge in the role of gene silencing through histone modifications in the maintenance of muscle phenotype and in regards to exercise training adaptations. In addition, Mdm2 and EZH2 interactions have not been examined in skeletal muscle or in the context of exercise training.

Hypothesis

We hypothesized that Mdm2 and EZH2 form a pathway that supports the maintenance and acquisition of an oxidative phenotype through the establishment of silencing marks on histones. Therefore, we postulate that the oxidative muscle phenotype is associated with a higher expression of EZH2 and Mdm2, which correlates with increased levels of H3K27me3.

Objectives

Here, we propose to characterize this putative Mdm2/EZH2/H3K27me3 pathway in the skeletal muscle at rest, during the adaptation process to long term and short term endurance training, and during C2C12 cell differentiation. In the context of this study, we will define endurance exercise as performing one bout of constant steady state running exercise on a treadmill. Short term training can be defined as the repetition of these bouts of endurance exercise daily from 1 day, up to 5 days. Long term training can be defined as the regular repetition of bouts of

endurance exercise for 9 weeks. This study has three main objectives. First, we will analyze the expression of Mdm2, EZH2, and the silencing mark H3K27me3 in phenotypically different skeletal muscles at basal levels. Next, we will investigate the impact of long term training on the abundance of Mdm2, EZH2, and H3K27me3 in the gastrocnemius, soleus, and plantaris. We will then test whether this pathway is sensitive to short term training. Lastly, we will examine the changes in abundance of Mdm2, EZH2, and H3K27me3 during C2C12 cell differentiation, as these cells adopt an oxidative phenotype in response to differentiation. If silencing marks are important in maintaining and acquiring of an oxidative phenotype, we expect to observe increases in EZH2, Mdm2, and H3K27me3.

Methods

Animal Studies

Ethical Approval

Animal training and care for the long term trained mice was done by students in Dr. Abdul-Sater's lab. Surgery to collect muscles and other samples were done by both students in our laboratory and Dr. Abdul-Sater's students. Animal training, care, and surgery for the short term trained mice was done by students in Dr. Haas' laboratory. Data collection for the present study was done by Manpreet Gulri. Some data was collected by Akash Mudur and Eric Chung. Dr. Abdul-Sater and Dr. Tara Haas received ethical approval by York University Committee on Animal Care, and studies were performed in accordance with Animal Care Procedures at York University.

Long Term Training

Fourteen female C57Bl6 mice, at 7 weeks old, were grouped into either the training (n=7) vs. sedentary group (n=7). All mice were fed the standard chow. Sedentary mice were allowed to roam their cages. Mice in the training group were acclimatized to treadmill exercise for 5 days at 20m/min prior to the nine-week training program. During the nine-week training program, mice ran for one hour per day, five days per week. The speed was gradually increased up to 30 m/min as the training program progressed: week one – 20 meters/min, week two - 22m/min, week three - 24.2m/min, week four - 26.6m/min, weeks five-nine - 30m/min. This represents speeds of 1.2 kmph to 1.8 kmph. The mice ran at approximately 70-75% of their VO2 max, which can be characterized as moderate to vigorous intensity. Mice were anaesthetized (isoflurane/oxygen inhalation) 72 hours after the last bout of exercise. Several lower limb muscles (including the

soleus, gastrocnemius, and plantaris) were removed, weighted, and snap-frozen in liquid nitrogen. The extensor digitorum longus (EDL) muscles were embedded in OCT on a spatula from tendon to tendon to maintain the structure of the muscle. EDL muscles were then frozen in isopentane and cooled by liquid nitrogen. The mice were euthanized by cervical dislocation. The gastrocnemius, soleus, and plantaris were utilized for protein and mRNA studies. The EDL muscle was utilized for immunohistochemistry.

Short Term Training

Sixteen female FVB/n mice, aged 9 weeks, purchased from Charles River (Saint-Constant, Qc, Canada). All mice were acclimatized to the treadmill (#91447-3 Columbus Instruments, Columbus, OH, USA) for 3 days prior to exercise training. Acclimatization proceeded by placing mice on the treadmill for 15 minutes at 15 m/min. The mice were randomly divided into 4 groups, where n=4 per group: sedentary, 1 day of training, 3 days of training, and 5 days of training. Mice in the exercise groups ran on the treadmill for 60 minutes at a speed of 25 m/min for each bout of training. To control for environment and handling, sedentary mice were placed on the treadmill daily. Prior to their final bout of exercise, mice were fasted for 4 hours. Mice were anaesthetized (isoflurane/oxygen inhalation) immediately after their final bout of training. The gastrocnemius, plantaris, and soleus muscles were extracted, weighed, and snap frozen in liquid nitrogen. The mice were euthanized by exsanguination (Slopack et al., 2014).

In Vitro Model

C2C12 Cell Differentiation

C2C12 myoblasts purchased from ATCC (product code: CRL-1772) were cultured onto 6-well plates with a loading density of 800,000 cells per well. The cells were coated with growth media (DMEM, with 10% fetal bovine serum (FBS Performance, Wisent), and 1% Penicillin-Streptomycin (10,000 U/mL) - Gibco). The cells were allowed to recover and adhere to the plates overnight in incubation at 37℃ for about 12 hours. Once the cells reached ~95% confluence, they were prepared for differentiation. The differentiation media (DMEM with 2% horse serum - Sigma-Aldrich: Horse Serum - Donor Herd, USA, and 1% penicillin/streptomycin solution) and 1X PBS (Phosphate Buffered Saline Solution, pH 7.4 (1X), Gibco) were warmed in a water bath. The growth media was aspirated and cells were washed with 1X PBS three times (2 mL per wash). 3 mL of differentiation media was added to each C2C12 cell culture well. The cells were placed in incubation at 37℃. The cells underwent differentiation over 5 days.

Experimental Analyses

Protein extraction from C2C12 cells

After each day of differentiation, one plate of cells was lysed using complete RIPA lysis buffer containing 50 mM Tris base, 100 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 5 mM EDTA, pH: 8.0 and complemented with 1mM PMSF protease inhibitor, phosphatase inhibitors (1 mM NaF, 1 mM Na_3VO_4), a protease inhibitor cocktail (Roche Diagnostics; cat # 049006845001) and a phosphatase inhibitor cocktail (Roche Diagnostics; cat # 11836153001).

Lysed cells were sonicated in the bioruptor at 4℃ for 5-8 cycles, 30 seconds on, 30 seconds off. The cells were then centrifuged for 15 minutes at 16,000 g to remove debris. Supernatant was collected and stored at -80℃.

Protein extraction from skeletal muscle

Protein from skeletal muscle samples was extracted using RIPA lysis buffer containing (50 mM Tris base, 100 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 5 mM EDTA, pH: 8.0) with 1mM PMSF protease inhibitor, phosphatase inhibitors (NaF, 1 mM, Na_3VO_4), a protease inhibitor cocktail (Roche Diagnostics; cat # 049006845001) and a phosphatase inhibitor cocktail (Roche Diagnostics; cat # 11836153001). Lysis was performed using the Retsch MM400 tissue lyser at 30 pulses/second for 30 seconds, and was repeated twice. Samples were placed on rotating agitation at 4℃ for 20 minutes. Samples were then placed in the biorupter for 8 cycles, 15 seconds on and 45 seconds off. Samples were placed in the centrifuge for 20 minutes at 12,000 g. Supernatant was collected and stored at -80℃.

Protein Concentration Determination

The bicinchoninic acid (BCA) assay was performed to determine total protein concentration of cell lysates and skeletal muscle extract. Cell protein extracts were diluted 1:5 with ddH20 and muscle protein extracts were diluted 1:20 with ddH2O. BCA working reagent was prepared with a bicinchoninic acid solution (Sigma-Aldrich; cat# 32 B9643) and copper (II) sulfate solution (Sigma-Aldrich; cat# C2284). Samples were incubated at 37℃ for 30 minutes before absorbance measurement at 562 nm (Gen5 plate reader, Biotek).

Western Blotting

Western blot analyses were conducted on protein extracts from mice gastrocnemius, soleus, and plantaris, as well as C2C12 cells. Blots were probed with the following primary antibodies: αβ-tubulin (Cell Signaling Technology; cat #2148), β-tubulin (Cell Signaling Technology; cat #2128), EZH2 (D2C9) XP (Cell Signaling Technology; cat #5246), Histone H2A II (Cell Signaling Technology; cat #2578), P-Mdm2 (S166) (Cell Signaling Technology; cat #3521), Mdm2 clone 2A10 (non-commercial), Tri-methyl-histone-H3 (K27) (C36B11) (Cell Signaling Technology; cat #9733), Histone H3 (96C10) (Cell Signaling Technology; cat #3638), Recombinant Anti-Histone H3 ubiquitinyl K23 antibody (Abcam; [EPR18876] ab194243), and β-actin (Santa Cruz Biotechnology; cat# sc-47778). Table 1.2 below summarizes the percentage of gel used for each primary antibody. After incubation with secondary antibody [horseradish peroxidase (HRP)-linked anti-rabbit antibody (Cell Signaling Technology; cat# 2148), HRP-linked anti-mouse antibody (Dako, cat# P0260); or light chain specific HRP-linked anti-mouse antibody (Cell Signaling Technology; cat#55802), proteins were visualized with enhanced chemiluminescence (ThermoFisher Scientific or Millipore) on Imaging Station 4000MM Pro (Carestream Health) or X-ray film (CL-XPosure Film, #34090). Blots were analyzed using the Carestream software.

Table 1.2. Primary antibodies and corresponding percentage gels used during electrophoresis for western blot analysis.

Primary Antibody	% of gel for electrophoresis
Histone H2A II	15%
P-Mdm2 (S166) and Mdm2 clone 2A10	10% or 6%
Tri-methyl-histone-H3 (K27)	15%
Histone H3	15%
Recombinant Anti-Histone H3 ubiquitinyl K23 antibody	15%
EZH2 and p-EZH2	10%

Immunoprecipitation

Previously collected C2C12 cell lysates were pooled and H3K27me3 was immunoprecipitated by overnight incubation using Protein A Magnetic Beads (Cell Signaling Technology; cat #73778). Normal Rabbit IgG (Cell Signaling Technology; cat #2729) was utilized as the primary antibody for species-specific control. Supernatants were analyzed by Western Blot for Ubiquitin (Abcam; cat#ab7780).

Immunohistochemistry

Immunohistochemistry was performed to determine the capillary-to-fiber ratio in the EDL muscle, which would reveal if angiogenesis had occurred. EDL tissue samples of sedentary and long term trained mice were utilized for immunohistochemistry. Samples were fixated onto slides using 3.7% formalin (1:100 ratio of 37% formaldehyde to PBS). or pure acetone. The samples were incubated for 30 minutes in the primary antibody CD31 (#77699S; Cell Signalling,

Danvers, MA, USA). This was followed by a wash in PBS. The samples were then incubated in secondary antibody (#32054; Thermo Fisher Scientific, Rockford, IL, USA). Following washing, samples were incubated in ABC reagent (Reagent A (#32054; Thermo Fisher Scientific, Rockford, IL, USA) to 10 mL PBS, then 180 µL of Reagent B (#32054; Thermo Fisher Scientific, Rockford, IL, USA) for 30 minutes, then washed with PBS. The slides were then submerged vertically in DAB solution for 5 minutes to stain, prepared from 5 µL DAB substrate (#1856090; Thermo Fisher Scientific, Rockford, IL, USA) in 45 µL DAB buffer (#1855910; Thermo Fisher Scientific, Rockford, IL, USA) (1:100). Permount mounting medium (#SP15-100; Thermo Fisher Scientific, Rockford, IL, USA) was applied around tissue samples. A cover slip was then added, and imaging was done using a Hamamatsu camera and Lambda 10-2 shutter. Capillary-to-fiber ratio was determined.

Statistical Analyses

Prism8 (GraphPad, San Diego, CA, USA) was utilized to perform all statistical analyses in this study. Statistical tests utilized were: the Student's *t*-test, one-way ANOVA, Bonferroni *post hoc* tests, Fisher's LSD and Dunnett multiple comparisons tests. All graphs plot mean ± SEM. Specific statistical information will be indicated in the figure legends of each figure.

Results

In Vivo Studies:

The silencing mark H3K27me3 appears at two molecular weights: 17 kDa and 25 kDa

First, we tested our capacity to measure silencing marks in the skeletal muscle. Gastrocnemius muscles of sedentary C57Bl6 mice were analyzed to measure protein expression of H3K27me3, which has an expected molecular weight of 17 kDa. When probing for H3K27me3 by Western Blot, another unknown band appeared at approximately 25 kDa, corresponding to a shift of 8 kDa (Figure 2.1A). Probing for H3 in the gastrocnemius muscles also revealed this upper band (Figure 2.1B). Both bands will be investigated in many of the upcoming results.

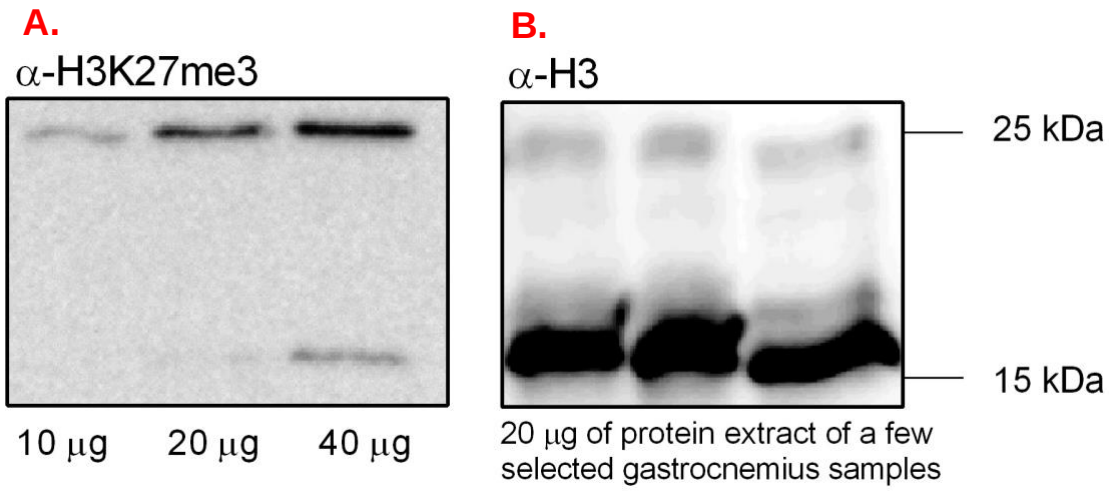


Figure 2.1 Shift in molecular weight was observed when probing for α-H3K27me3 and α-H3.

The samples analyzed were from C57Bl6 female gastrocnemius muscles. In A, the samples were pooled and in B, a few select gastrocnemius samples were chosen. Muscles were extracted when mice were 14 weeks of age. **A.** shows a representative western blot for H3K27me3 protein levels. Different amounts of protein extract of pooled gastrocnemius muscle were tested: 10, 20, and 40 µg. **B.** shows a representative western blot for H3 protein levels. 20 µg of protein extract of a few selected sedentary gastrocnemius muscles were tested.

The relative abundance of Mdm2, EZH2, and H3K27me3 differs in highly oxidative muscles

We have analyzed the soleus, gastrocnemius, and plantaris muscles, which are phenotypically different to test whether the abundance of H3K27me3, Mdm2, and EZH2 are dependent on muscle phenotype. The soleus muscle is highly oxidative, the gastrocnemius muscle is mixed, and the plantaris muscle is highly glycolytic. Prior to observing the effects of exercise training, we investigated basal levels of the proteins of interest in the mentioned skeletal muscles of sedentary 14-week-old mice. The soleus muscle expressed 86% and 73% more Mdm2 protein than the gastrocnemius and plantaris muscle respectively. No differences were observed between the plantaris and gastrocnemius (Figure 2.2A). In contrast, the soleus muscle expressed about 82% less protein levels of EZH2 compared to the plantaris and gastrocnemius muscles. There was no difference in EZH2 protein levels between the plantaris and the gastrocnemius (Figure 2.2B). Similar to the observation made regarding the change in Mdm2, we found that protein expression of H3K27me3 at 17 kDa was increased by 68% and 88% in the soleus when compared to the plantaris and the gastrocnemius. There was no difference in H3K27me3 (17 kDa) levels between the gastrocnemius and plantaris (Figure 2.3A). Similarly, H3K27me3 at 25 kDa protein levels were about 85% higher in the soleus muscle compared to the plantaris and gastrocnemius (Figure 2.3B). These data highlight the absences of differences between the gastrocnemius and the plantaris muscles.

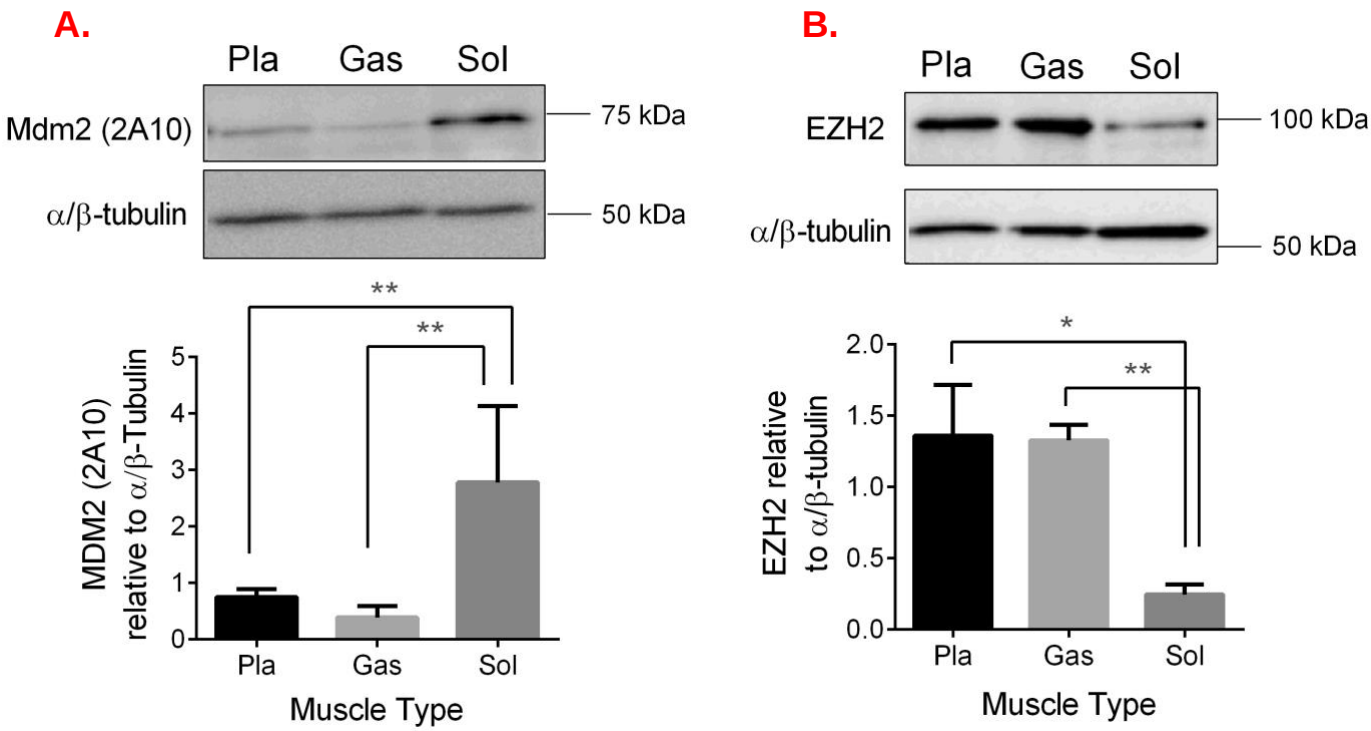


Figure 2.2 Basal levels of Mdm2 and EZH2 differ between muscle types in mice.

The samples analyzed were from C57Bl6 female sedentary mice. Muscles were extracted when mice were 14 weeks of age. Plantaris (Pla), gastrocnemius (Gas), and soleus (Sol) muscles were tested. **A.** shows Mdm2 protein levels relative to α/β-Tubulin. **B.** shows EZH2 protein levels relative to α/β-Tubulin. The molecular weight represents the nearest marker to the molecular weight of the protein. Significant effect ($p<0.05$) (one way ANOVA) *=$p<0.05$. (Tukey's multiple comparison). n=5 per group. Data presented as: mean ± SEM.

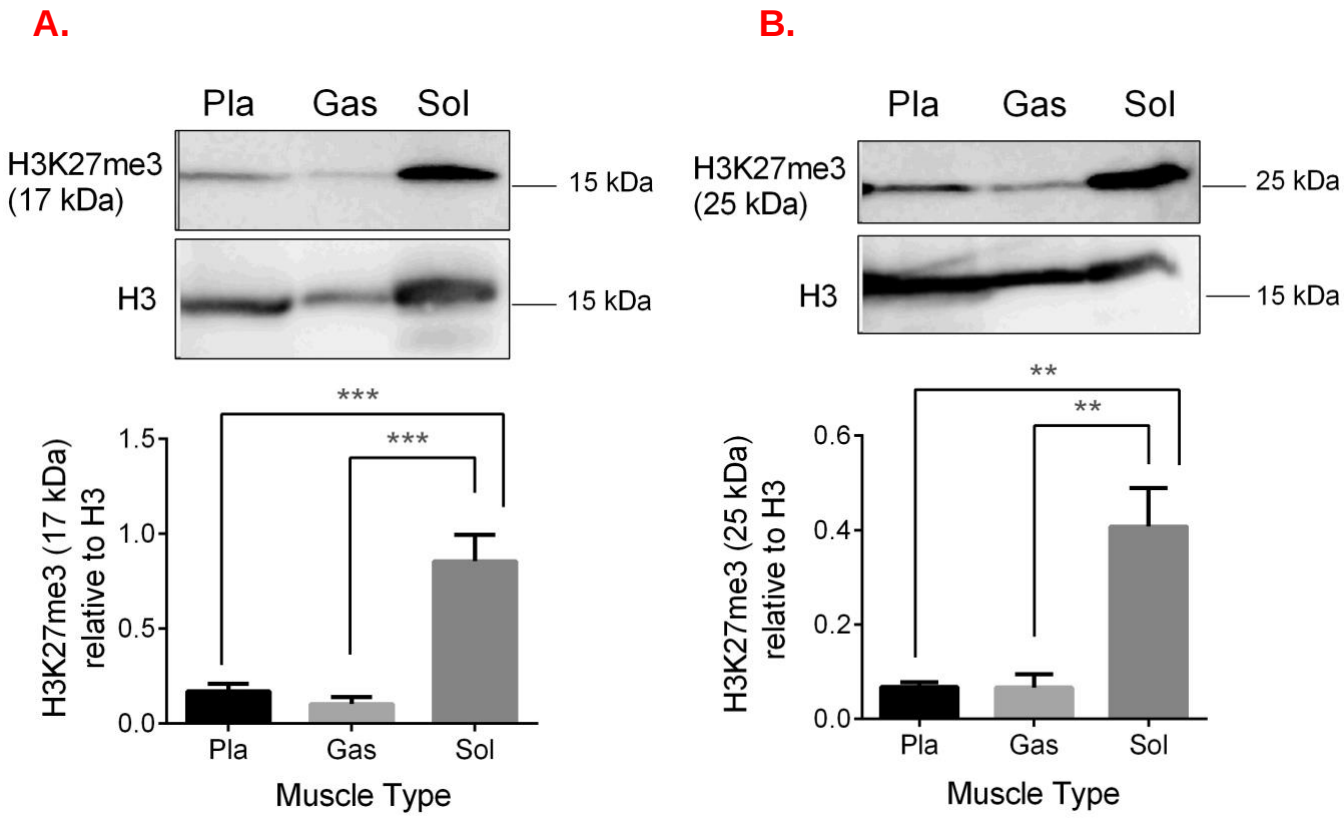


Figure 2.3. Basal levels of H3K27me3 at 17 and 25 kDa differ among muscle types in mice.

The samples analyzed were from C57Bl6 female sedentary mice. Muscles were extracted when mice were 14 weeks of age. Plantaris (Pla), gastrocnemius (Gas), and soleus (Sol) muscles were tested. **A.** shows H3K27me3 (17 kDa) protein levels relative to H3. **B.** shows H3K27me3 (25 kDa) protein levels relative to H3. The molecular weight represents the nearest marker to the molecular weight of the protein. Significant effect ($p<0.05$) (one way ANOVA) *=$p<0.05$. (Tukey's multiple comparison). n=5 per group. Data presented as: mean ± SEM.

Long Term Training Effects in the Skeletal Muscle of Mice

The observations made at basal levels suggested that there was differential regulation of EZH2, Mdm2, and H3K27me3 to maintain muscle phenotype of different muscles. Endurance training induces a shift towards a more vascular and oxidative phenotype in skeletal muscle (Lee et al., 2017). Therefore, we tested how nine weeks of endurance training would affect our proteins of interest in mice. First, we confirmed the efficacy of the long term training by measuring the capillary-to-fiber (C:F) ratio in the extensor digitorum longus (EDL) muscle. C:F ratio in the EDL muscle showed a significant increase in trained mice compared to sedentary (n=6 per group, trained: 1.22 ± 0.046 vs. sedentary: 1.00 ± 0.036 [Means ± SEM], p=0.0036). This unpublished data was obtained by an undergraduate student from our laboratory.

The protein levels of COX IV in the gastrocnemius were also used as a measure for the efficacy of the long term training program. COX IV protein levels significantly increased due to nine weeks of training (n=7 per group, trained: 1.20 ± 0.178 vs. sedentary: 0.50 ± 0.148 [Means ± SEM], p=0.0110). Since angiogenesis is a major adaptation to exercise training, Mdm2 protein levels were of interest considering the importance of Mdm2 during this process. Mdm2 protein levels significantly increased in the gastrocnemius of mice that underwent nine weeks of endurance training (Figure 2.4A).

EZH2 protein levels were significantly higher in the gastrocnemius muscles of trained mice compared to sedentary mice (Figure 2.5A). No effect of training was observed in EZH2 protein levels in the plantaris muscle between trained and sedentary mice (Figure 2.5B). In the soleus muscle, there appeared to be a non-significant increase in EZH2 protein levels with training (Figure 2.5C).

The protein levels of H3K27me3 at 17 kDa did not change in the gastrocnemius and soleus muscles (Figure 2.6A, Figure 2.6C). However, in the plantaris muscle, endurance training caused a significant decrease in H3K27me3 (17 kDa) protein levels (Figure 2.6B). Interestingly, endurance training caused an increase in H3K27me3 at 25 kDa in the gastrocnemius muscle in mice (Figure 2.7A).

We have shown in previous figures that when probing for H3K27me3, there was about an 8 kDa increase in molecular weight. This corresponds to the size of a mono-ubiquitin modification. Therefore, we were interested in measuring the abundance of histone ubiquitination. We measured the protein levels of ubiquitinated H3K23 (H3K23UB) in sedentary and endurance trained mice. Endurance training did not cause any significant change in H3K23Ub in the gastrocnemius muscle in mice (Figure 2.8A).

Changes in Mdm2

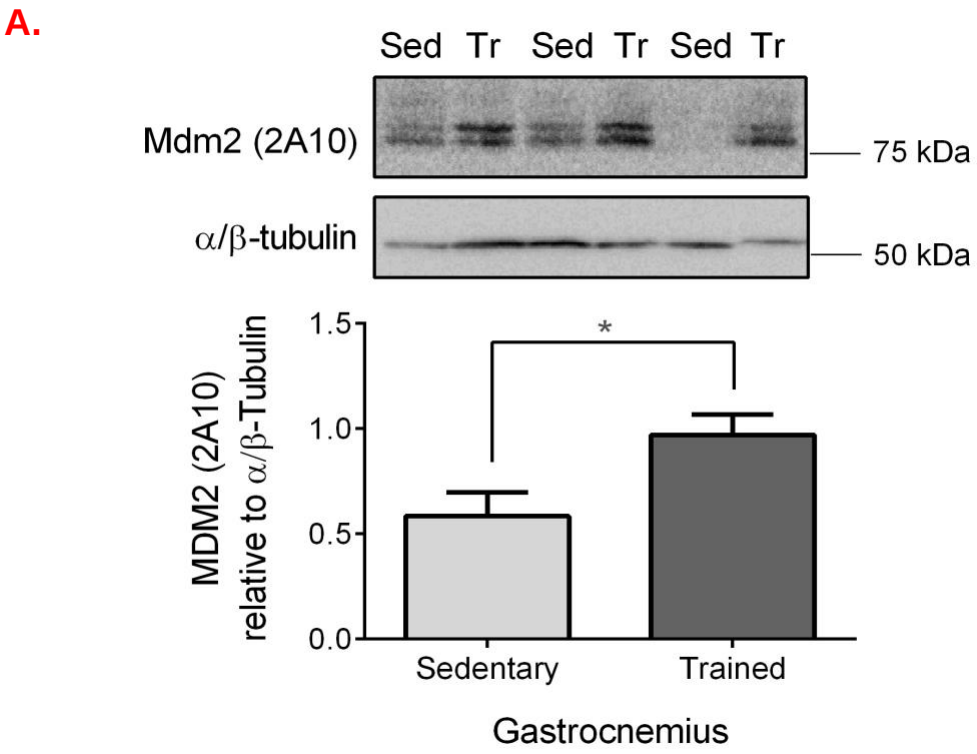


Figure 2.4. Long term training elicits an increase in Mdm2 protein levels in the gastrocnemius muscle of mice

The samples analyzed were from C57Bl6 female mice. Mice underwent endurance training for 9 weeks or were sedentary. Muscles were extracted when mice were 14 weeks of age and 72 hours after the last bout of training. The gastrocnemius muscle tested. **A.** Mdm2 protein levels relative to α/β-Tubulin in the gastrocnemius muscle. The molecular weight represents the nearest marker to the molecular weight of the protein. Significant effect ($p<0.05$) (Student's t-test) *=$p<0.05$. n=7 per group. Data presented as: mean ± SEM.

Changes in EZH2

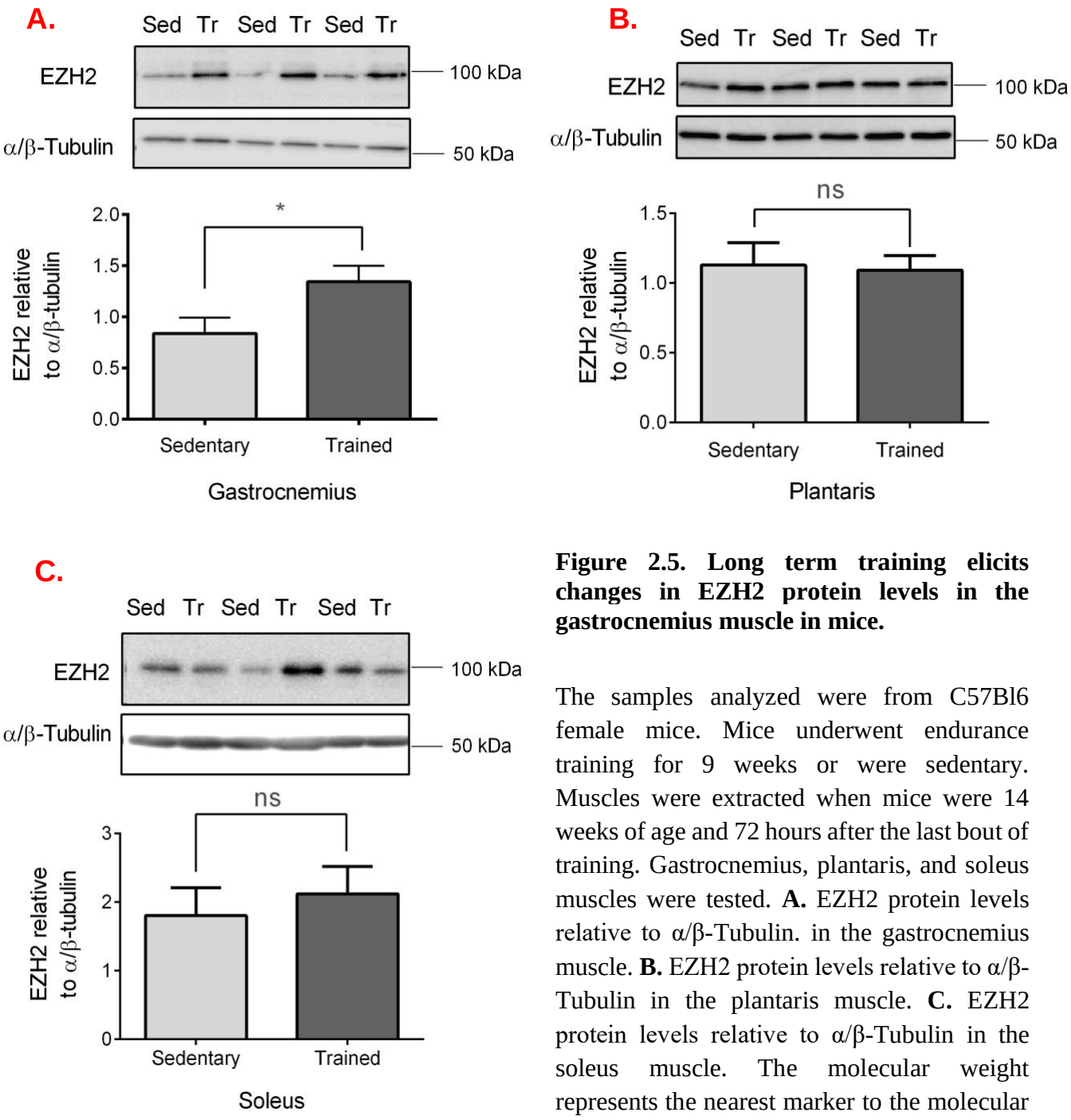


Figure 2.5. Long term training elicits changes in EZH2 protein levels in the gastrocnemius muscle in mice.

The samples analyzed were from C57Bl6 female mice. Mice underwent endurance training for 9 weeks or were sedentary. Muscles were extracted when mice were 14 weeks of age and 72 hours after the last bout of training. Gastrocnemius, plantaris, and soleus muscles were tested. **A.** EZH2 protein levels relative to α/β-Tubulin. in the gastrocnemius muscle. **B.** EZH2 protein levels relative to α/β-Tubulin in the plantaris muscle. **C.** EZH2 protein levels relative to α/β-Tubulin in the soleus muscle. The molecular weight represents the nearest marker to the molecular weight of the protein. Significant effect ($p<0.05$) (Student's t-test) *=$p<0.05$. n=7 per group. Data presented as: mean ± SEM.

Changes in H3K27me3 at 17 kDa

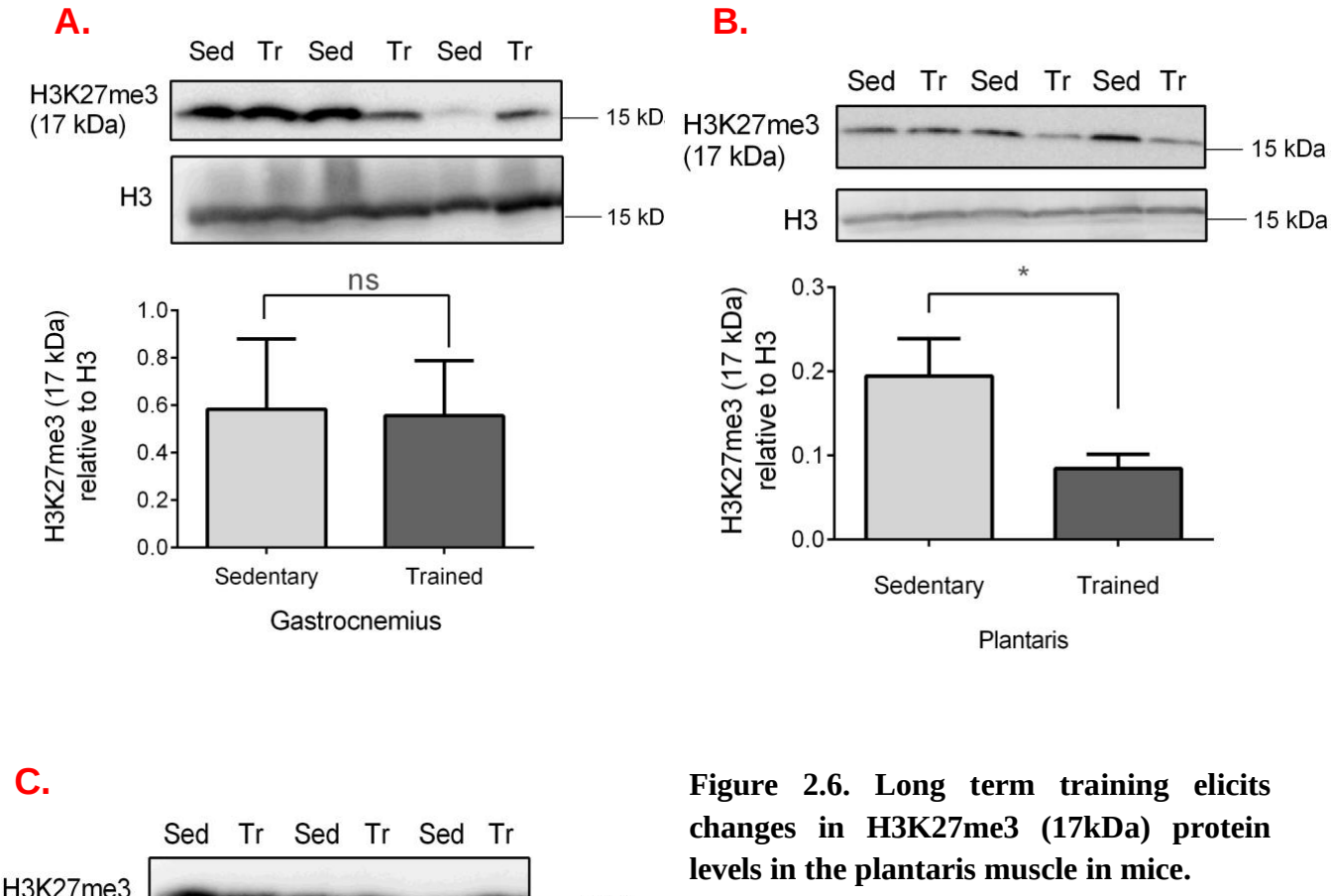


Figure 2.6. Long term training elicits changes in H3K27me3 (17kDa) protein levels in the plantaris muscle in mice.

The samples analyzed were from C57Bl6 female mice. Mice underwent endurance training for 9 weeks or were sedentary. Muscles were extracted when mice were 14 weeks of age and 72 hours after the last bout of training. Gastrocnemius, plantaris, and soleus muscles were tested. **A.** H3K27me3 (17 kDa) protein levels relative to H3 in the gastrocnemius muscle. **B.** H3K27me3 (17 kDa) protein levels relative to H3 in the plantaris muscle. **C.** H3K27me3 (17 kDa) protein levels relative to H3 in the soleus muscle. The molecular weight represents the nearest marker to the molecular weight of the protein. Significant effect ($p<0.05$) (Student's t-test) *=$p<0.05$. n=7 per group. Data presented as: mean ± SEM.

Changes in H3K27me3 (25 kDa)

A.

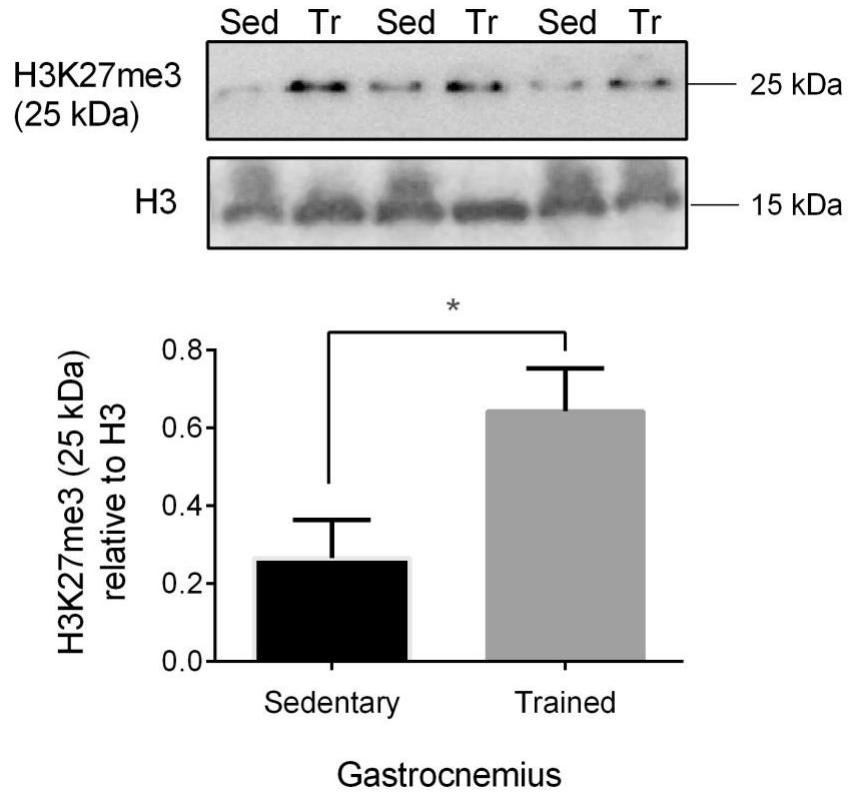


Figure 2.7. Long term training elicits an increase in H3K27me3 (25 kDa) protein levels in the gastrocnemius muscle of mice.

The samples analyzed were from C57Bl6 female mice. Mice underwent endurance training for 9 weeks or were sedentary. Muscles were extracted when mice were 14 weeks of age and 72 hours after the last bout of training. The gastrocnemius muscle tested. **A.** H3K27me3 (25 kDa) protein levels relative to H3 in the gastrocnemius muscle. The molecular weight represents the nearest marker to the molecular weight of the protein. Significant effect ($p<0.05$) (Student's t-test) *=$p<0.05$. n=7 per group. Data presented as: mean ± SEM.

Changes in H3K23Ub

A.

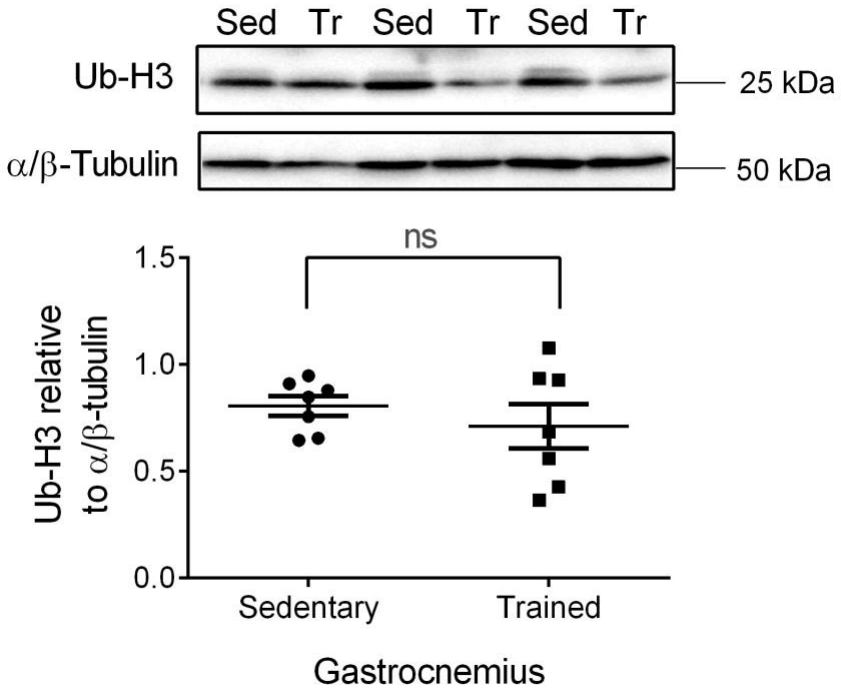
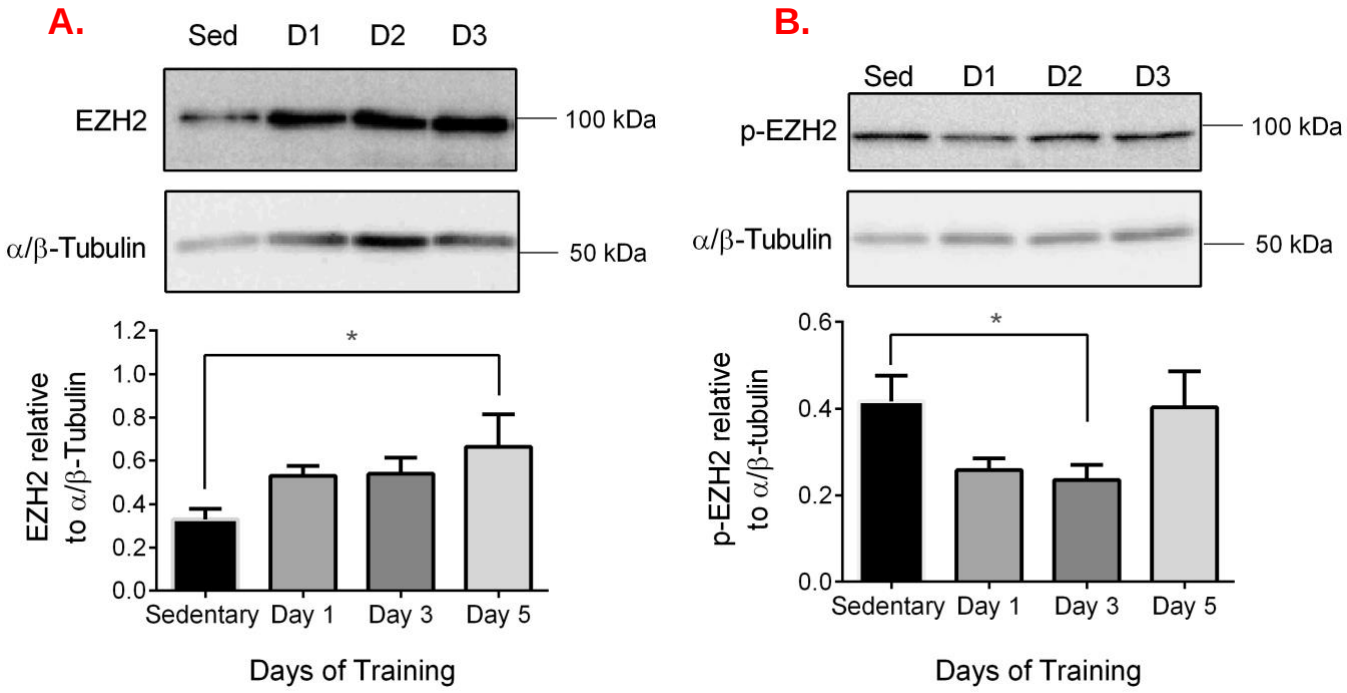


Figure 2.8. Long term training does not change H3K23Ub protein levels in the gastrocnemius muscle of mice.

The samples analyzed were from C57Bl6 female mice. Mice underwent endurance training for 9 weeks or were sedentary. Muscles were extracted when mice were 14 weeks of age and 72 hours after the last bout of training. The gastrocnemius muscle tested. **A.** H3K23Ub protein levels relative to α/β-Tubulin in the gastrocnemius muscle. The molecular weight represents the nearest marker to the molecular weight of the protein. No significant effect was reported (p=0.4190, Student's t-test, n=7 per group). Data presented as: mean ± SEM.

Short-Term Training Effects in the Plantaris Muscle of Mice

Due to the high stress conditions that occur during the first few repeated bouts of exercise, many adaptations begin to take place during this period. We know that an acute bout of exercise induces changes in the activity of AMPK, which is known to phosphorylate EZH2 on Threonine 311 (pEZH2-Thr311). Therefore, we have measured whether daily repetitions of bouts of exercise during short term training impact this phosphorylated form of EZH2, which has been reported to be phosphorylated by AMPK. The effects of short-term training were observed in the plantaris muscle of female FVB/n mice. We found that EZH2 protein levels significantly increased, with the highest levels found at Day 5 of training (Figure 2.9A). Surprisingly, p-EZH2 protein levels were found to significantly decrease by Day 3 of training. There was an increase in p-EZH2 levels seen at Day 5, however, this was not significant (Figure 2.9B). When analyzing the ratio of p-EZH2 to EZH2, we found that there was a significant decrease at all exercise training time points (Figure 2.9C).



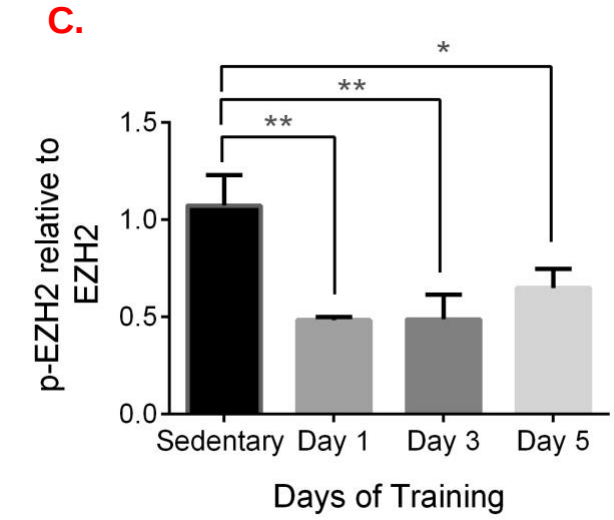


Figure 2.9. Short term training elicits changes in EZH2 and p-EZH2 (Thr311) protein levels in the plantaris muscle in mice.

FVB/n female mice were either sedentary or exercised daily for 5 days. Plantaris muscles were extracted immediately after the bout of exercise. **A.** EZH2 protein levels relative to α/β-Tubulin in the plantaris muscle. **B.** p-EZH2 (Thr311) protein levels relative to α/β-Tubulin in the plantaris muscle. **C.** p-EZH2 (Thr311) protein levels relative to EZH2 in the plantaris muscle. The molecular weight represents the nearest marker to the molecular weight of the protein. Significant effect ($p<0.05$) (one way ANOVA) *=$p<0.05$. (Fisher's test for multiple comparisons). n=4 per group. Data presented as: mean ± SEM.

In Vitro Studies:

C2C12 Differentiation

Endurance training changes the expression of protein levels of Mdm2, EZH2, and H3K27me3 (17 kDa and 25 kDa), as seen in previous figures. In order to further investigate the role of EZH2, Mdm2 and H3K27me3 (17 kDa and 25 kDa) in the process of myogenesis, we analyzed how the expression of these proteins and their related silencing marks were modulated during C2C12 differentiation over 5 days. As expected, we found that Mdm2 protein levels significantly increased during C2C12 differentiation. The increase in Mdm2 was observed as early as day 2 of differentiation, and showed the most pronounced increase by day 4 (Figure 2.10A).

In agreement with previous studies, EZH2 protein levels significantly decreased during C2C12 differentiation, and were almost depleted by day 5 (Figure 2.11A). Interestingly, another unknown band was observed above the expected EZH2 band. This band showed the opposite effect of EZH2 upon differentiation. There was a significant increase in the protein levels of the unknown band during differentiation, with the most pronounced increase by day 4 (Figure 2.11B).

During the process of C2C12 differentiation, the abundance of H3K27me3 (17 kDa) increased over time ($p=0.0343$, overall effect of time of differentiation). Indeed, in undifferentiated C2C12 cells, the abundance of H3K27me3 (17 kDa) is minimal, however, this peaked at day 2 where differentiating C2C12 cells showed higher levels of the silencing mark than undifferentiated cells ($p \leq 0.05$) (Figure 2.12A). It is important to note that high variability was observed when measuring H3K27me3 during C2C12 differentiation. H3K27me3 at 25 kDa showed a steady increase, which reached significance by day 4 (Figure 2.12B). The data were highly variable at day 5 and somewhat variable at day 4.

To observe whether H3K27me3 was bound to ubiquitin, we co-immunoprecipitated H3K27me3 in C2C12 protein extract. The C2C12 protein extract from both undifferentiated and differentiated cells was pooled together, immunoprecipitated and probed for ubiquitin by immunoblotting. We found that ubiquitin was indeed bound to H3K27me3 (Figure 2.13A).

Changes in Mdm2

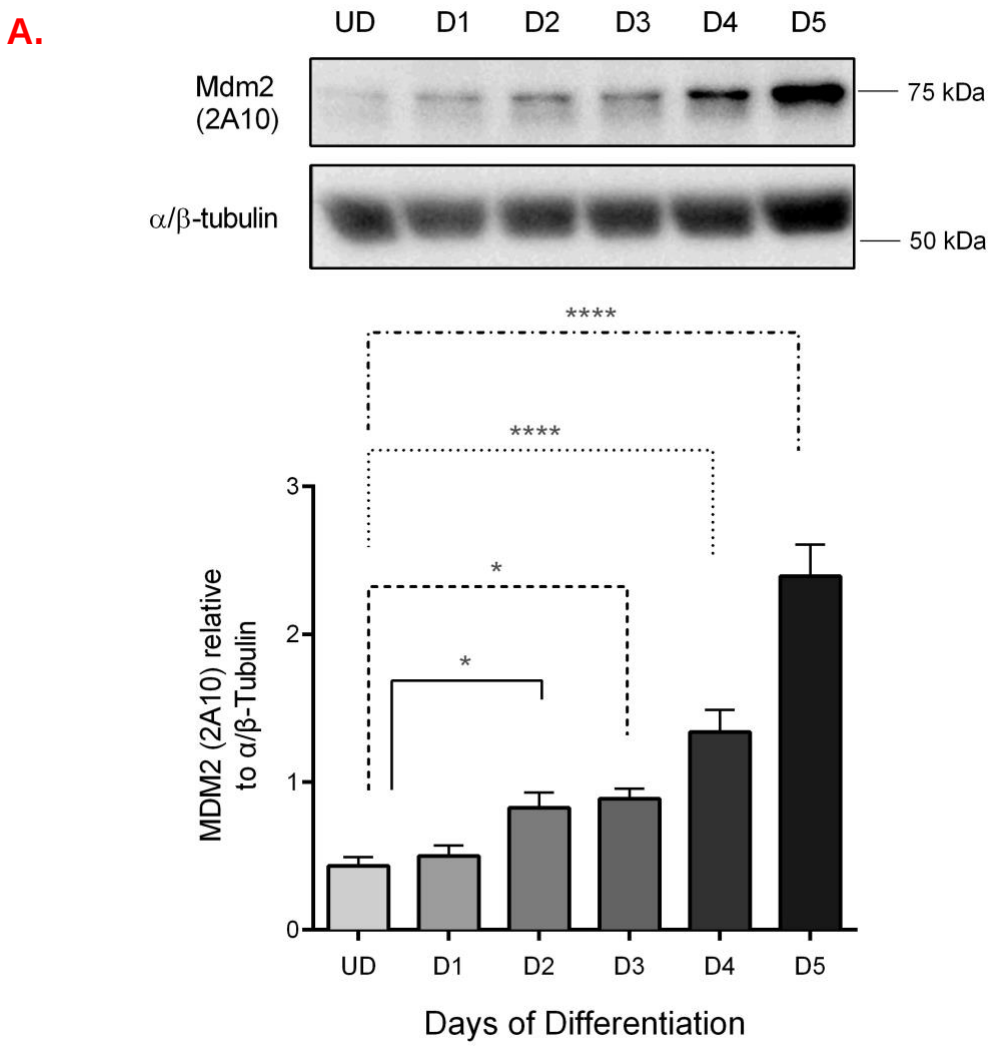


Figure 2.10. C2C12 differentiation leads to a significant increase in Mdm2 protein levels. C2C12 myoblasts were differentiated over 5 days and extracted after each day of differentiation. **A.** Mdm2 protein levels relative to α/β-Tubulin in C2C12 differentiating myoblasts. The molecular weight represents the nearest marker to the molecular weight of the protein. Significant effect ($p<0.05$) (one way ANOVA) *=$p<0.05$. (Fisher's test for multiple comparisons). n=6 per group. Data presented as: mean ± SEM.

Changes in EZH2

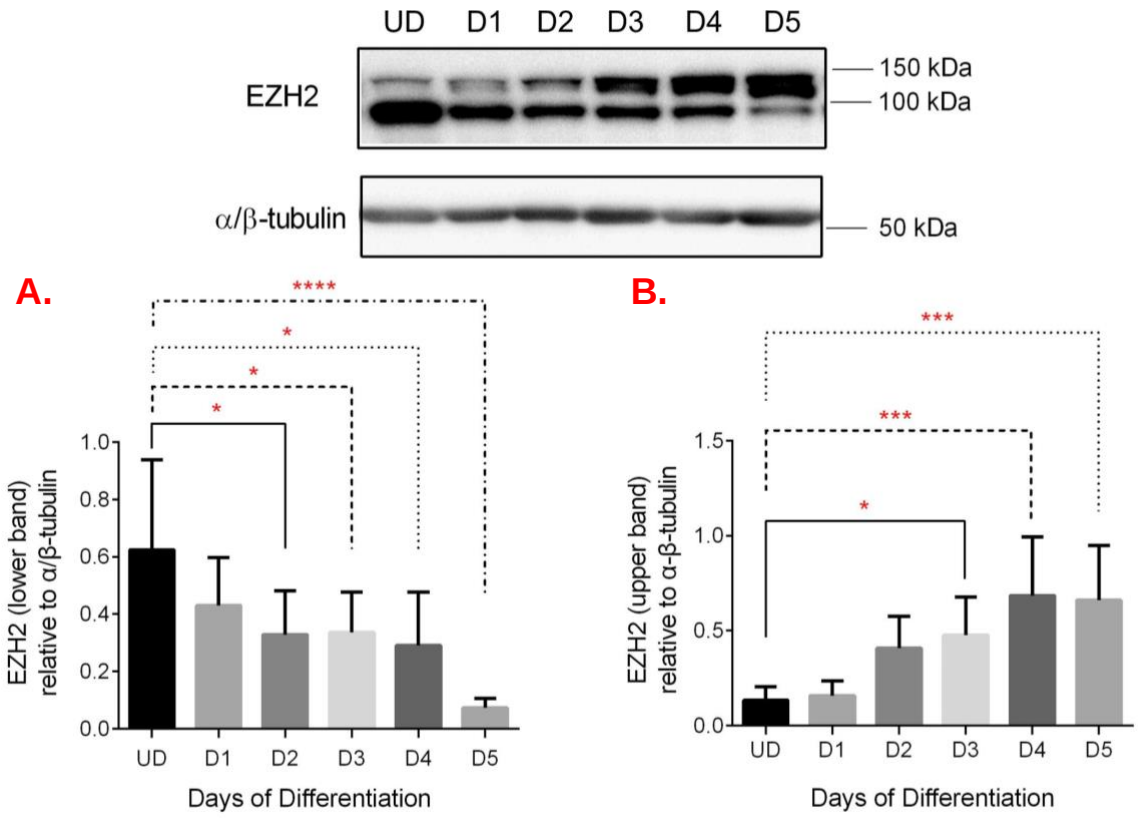


Figure 2.11. C2C12 differentiation leads to a significant depletion of EZH2 protein levels and an increase in the upper band.

C2C12 myoblasts were differentiated over 5 days and extracted after each day of differentiation. **A.** shows EZH2 protein levels relative to α/β-Tubulin in C2C12 differentiating myoblasts. **B.** shows the upper band detected by EZH2 antibody. Protein levels relative to α/β-Tubulin in C2C12 differentiating myoblasts. The molecular weight represents the nearest marker to the molecular weight of the protein. Significant effect ($p<0.05$) (one way ANOVA) *=$p<0.05$. (Fisher's test for multiple comparisons). n=6 per group. Data presented as: mean ± SEM.

Changes in H3K27me3

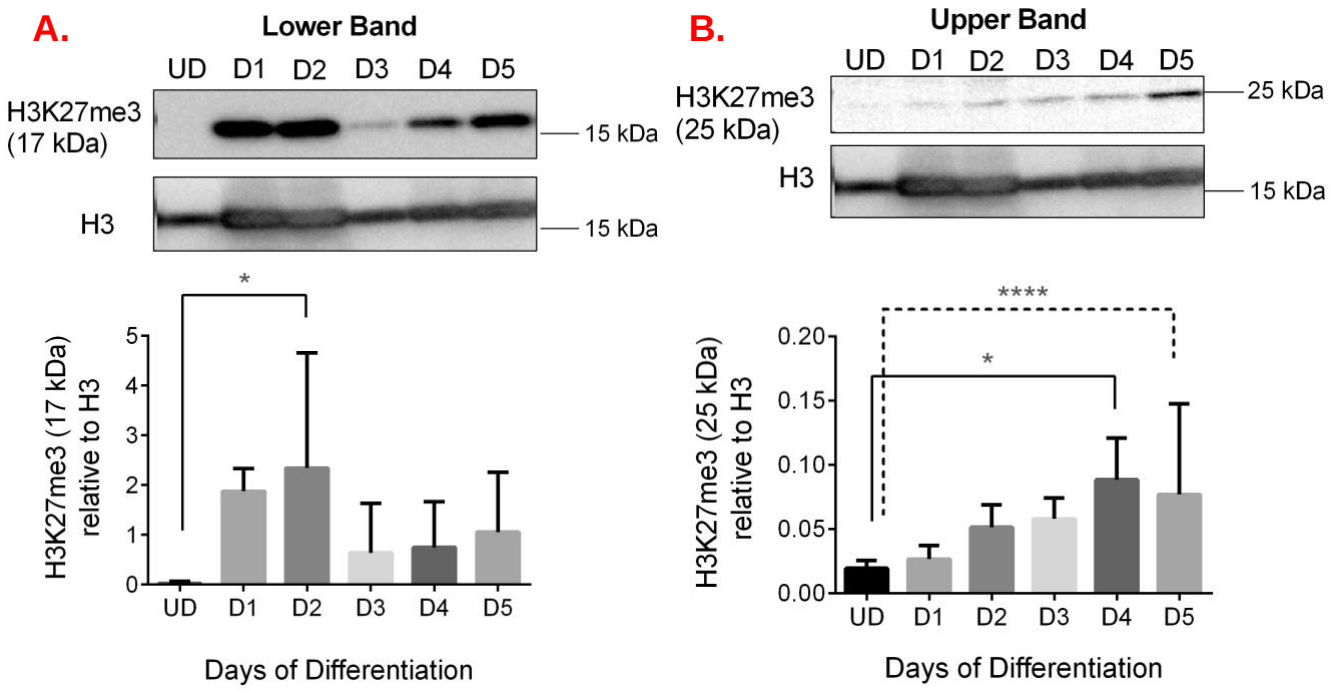


Figure 2.12. C2C12 differentiation leads to a significant increase in H3K27me3 both at 17 kDa and 25 kDa.

C2C12 myoblasts were differentiated over 5 days and extracted after each day of differentiation. **A.** shows H3K27me3 (17 kDa) protein levels relative to H3 in C2C12 differentiating myoblasts. **B.** shows H3K27me3 (25 kDa) protein levels relative to H3 in C2C12 differentiating myoblasts. The molecular weight represents the nearest marker to the molecular weight of the protein. Significant effect of differentiation ($p<0.05$) (one way ANOVA) *=$p<0.05$. (Fisher's test for multiple comparisons). n=6 per group. Data presented as: mean ± SEM.

H3K27me3 and ubiquitin are bound in C2C12 Cells

A.

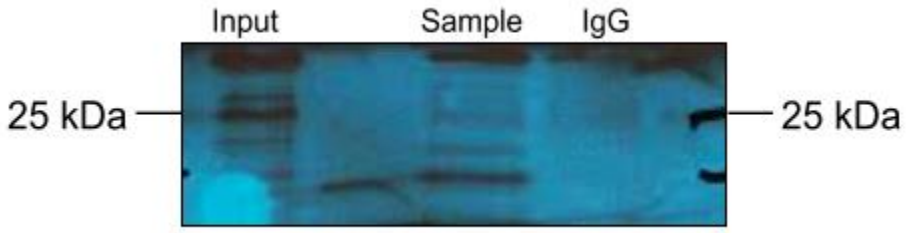


Figure 2.13. Immunoprecipitation reveals that ubiquitin is bound to H3K27me3 in C2C12 cells.

C2C12 myoblasts were differentiated over 5 days and extracted after each day of differentiation. The sample analyzed was protein extract produced by pooling together all samples. **A.** After immunoprecipitation (IP) of H3K27me3 from C2C12 pooled protein extract, levels of Ubiquitin were measured by immunoblot analysis in whole-cell lysate (input) and in the IP product (n=1).

Discussion

Our results support a potential correlation between silencing marks and the oxidative phenotype. This correlation provides evidence for a supportive role of silencing marks in maintaining the oxidative phenotype. At basal levels, the oxidative soleus muscle, showed higher protein abundance of the silencing mark, H3K27me3. In support of this, H3K27me3 (17 kDa) abundance increases during C2C12 differentiation, as these cells adopt a more oxidative phenotype. Mdm2 plays a role in maintaining silencing marks in stem and cancer cells, in addition to being a pro-angiogenic factor in the skeletal muscle. Mdm2 protein levels were significantly higher in the soleus muscle at basal levels, as expected. This also supports the idea that the pathway supporting silencing marks may be involved in the maintenance of the oxidative phenotype. Endurance training leads to adaptations in skeletal muscle that support an oxidative phenotype. Our results indicate that endurance training increases the abundance of EZH2 and Mdm2 in the gastrocnemius muscle. Unexpectedly, this was not correlated with an increased abundance of H3K27me3 at the expected molecular weight of 17 kDa. However, we detected an increased abundance of an upper shifted band at 25 kDa that appears when probing for H3K27me3. Further investigations and mechanistic studies need to be completed to understand the nature of Mdm2-EZH2 interactions and their role in establishing the silencing mark on histone 3 in the skeletal muscle.

The long term training program for the C57Bl6 mice showed some of the most robust changes in this study. We were able to confirm the efficacy of long term training by measuring capillary-to-fiber ratio and protein levels of COX IV, which both increased in trained mice. An increase in Mdm2 protein levels is another indication that the mice had adapted to training. This was confirmed in our study, as long term trained mice showed higher protein levels of Mdm2 in

the gastrocnemius muscle. This increase is in line with our previously published data, where endurance term training led to an increase in Mdm2 protein levels in the plantaris muscle of rats (Roudier et al., 2012). These results collectively reveal that the skeletal muscles were adapting towards a more oxidative phenotype. An increase in C:F ratio shows that capillarization has increased in the muscle, allowing for more blood flow to working muscle. Additionally, the increase in COX IV indicates increased mitochondrial capacity (Bengtsson et al., 2001).

We have found that two bands appear when probing for H3K27me3: one at 17 kDa (the standard molecular weight) and one at 25 kDa. The oxidative and well-vascularized soleus muscle shows higher abundance of both bands for H3K27me3, and higher levels of Mdm2 protein compared to the more glycolytic and less vascularized muscles, the gastrocnemius and plantaris at basal levels. Surprisingly, the soleus showed reduced levels of EZH2 despite higher abundance of H3K27me3. Repeated bouts of exercise (short term training) caused a gradual increase in protein levels of EZH2. However, this was found to be associated with reduced phosphorylation of EZH2 at Thr311 in the plantaris. This suggests that short term training supports EZH2 function in the early stages of exercise training. However, during long term training, we found reduced abundance of H3K27me3 (17 kDa) in the plantaris. Long term training changes the abundance of H3K27me3 and EZH2 in a muscle-specific manner. The gastrocnemius shows an increase in both Mdm2 and EZH2, which is associated with an increase in H3K27me3 at 25 kDa only.

Satellite cells, which are precursors to skeletal muscle cells, can be activated during endurance training (Bazgir et al., 2017). This provided a rationale for studying the modulation of Mdm2, EZH2, and H3K27me3 during C2C12 differentiation. C2C12 differentiation led to an overall increase in Mdm2, H3K27me3 (both 17 and 25 kDa), and a decrease in EZH2 protein

levels. Interestingly, an upper band was detected when probing for EZH2. This band showed increased protein abundance as differentiation proceeded.

H3K27me3 has a standard molecular weight at 17 kDa. Our study has revealed that when probing for H3K27me3, an additional upper band appears at 25 kDa. At first, we thought the cause of this was non-specific binding, a common issue during Western Blotting. When probing for H3K27me3, the standard 17 kDa band showed a weak signal, whereas the 25 kDa was the predominant band. To obtain a detectable and well-quantifiable signal at 17 kDa, we had to use more protein extract (40 µg). We were studying very small muscles, such as the plantaris and soleus, and this process was using a lot of protein extract. To limit loss of extract, we cut the membrane below the 25 kDa band prior to adding the primary antibody on the remaining bottom part of the membrane. This significantly increased the capacity to detect H3K27me3 at 17 kDa. Hence, the upper band was largely ignored in the early stages of my study. Upon further research, we decided to test this band for possible post-translational modifications (PTMs). Most PTMs, such as methylation, are small enough to maintain the molecular weight of the protein. However, we noticed that the upper band appeared at approximately 25 kDa - about 8 kDa larger than the standard H3K27me3 protein weight. Mono-ubiquitination has been shown in other studies to increase the molecular weight by approximately 8 kDa (Zhang et al., 2017). In fact, Zhang et al. have shown that H3, can be mono-ubiquitinated on several lysine residues including K9, K14, K23, and K27, by the E3 ubiquitin ligase NEDD4 upon glucose stimulation. These data provided further evidence that the H3K27me3 upper band may have been the result of mono-ubiquitination. These data were of particular interest, since our study was also observing an E3 ubiquitin ligase: Mdm2.

Zhang et al. revealed that glucose-induced mono-ubiquitination regulates H3 acetylation. More specifically, mono-ubiquitination was required for the acetylation of H3K9 and H3K14 (Zhang et al., 2017). This suggests that mono-ubiquitination may be a mechanism to remove silencing marks on genes in order to ease transcriptional activation. In the context of exercise training, previous studies have shown the importance of establishing activation marks on genes associated with the type I oxidative phenotype (McGee and Hargreaves, 2011). Therefore, it is important to investigate the potential mono-ubiquitination observed in this study, as it may play a role in the reactivation of genes related to type I oxidative fibers.

Our previous work has shown that skeletal muscles with higher levels of capillarization, such as the soleus, have higher levels of Mdm2 protein expression (Roudier et al., 2012). The present study supports this data, as in mice Mdm2 protein levels were higher in the soleus muscle, compared to the mixed and fast twitch gastrocnemius and plantaris muscles, respectively. EZH2 protein levels revealed the opposite effect compared to Mdm2. EZH2 protein levels were higher in the gastrocnemius and plantaris muscles than in the soleus. These data can be explained and complemented by the C2C12 differentiation study. As C2C12 cells differentiate, they adopt a more oxidative phenotype and EZH2 is depleted (Caretti et al., 2004; Wagatsuma and Sakuma, 2013). Indeed, our study supported this as we observed EZH2 depletion during C2C12 differentiation. This suggests that when a muscle is more oxidative, EZH2 is not required to maintain the phenotype. Interestingly, the glycolytic muscles show higher levels of EZH2. During C2C12 differentiation, we observed that undifferentiated cells or cells at the early stages of differentiation, show high levels of EZH2. During these stages, the cells are more glycolytic. These similarities suggest that glycolytic tissues require more gene silencing through EZH2 than oxidative tissues.

Protein levels of H3K27me3 (17 kDa) were higher in the oxidative soleus muscle compared to the gastrocnemius and plantaris muscles. This indicates that there is a greater abundance of silencing marks present on oxidative muscle. As discussed above, EZH2 levels were observed to be significantly lower in the soleus muscle. There is a possibility that EZH2 is no longer needed in the soleus muscle, since the silencing marks are already in place. We hypothesize that once EZH2 has established silencing marks, Mdm2 could serve as an E3 ubiquitin ligase to reduce EZH2 protein levels through degradation. However, this hypothesis will require further investigation. H3K27me3 at 25 kDa, the putative mono-ubiquitination mark, showed significantly higher protein levels in the soleus muscle compared to the gastrocnemius and the plantaris. If the 25 kDa band is indeed mono-ubiquitinated H3K27me3, then the higher levels of this protein can be explained by the need to activate certain genes in order to maintain the oxidative phenotype. Since H3 ubiquitination has been shown to be required for H3 acetylation, as a way to ease activation, it is not surprising that the soleus shows higher levels of this potential PTM.

We found that long term trained mice showed higher protein levels of EZH2 in the gastrocnemius only. The plantaris and soleus muscle showed no significant change in EZH2 levels in response to long term endurance training. Since most training adaptations occur in the first few weeks of exercise, it is possible that EZH2 levels were elevated in the gastrocnemius to cause silencing of more genes. Presumably, the newly silenced genes would be those that are no longer needed since the adaptation process was previously completed.

Additionally, long term training led to the decrease in protein levels of the silencing mark H3K27me3 at 17 kDa in the plantaris muscle. However, there were no changes in the silencing mark observed in both the soleus and the gastrocnemius. On the other hand, the putative mono-ubiquitinated H3K27me3 at 25 kDa significantly increased as a result of long term training in the

gastrocnemius muscle. Unfortunately, we do not have the data to show the changes in the plantaris and soleus for this potential PTM due to long term training, as the membranes from western blotting were cut to avoid the 25 kDa band in the earlier stages of the project. However, it would be interesting to observe the protein levels of mono-ubiquitinated H3K27me3 in the plantaris muscle, since the levels of the silencing mark decrease. The plantaris is a fast-twitch muscle and relies highly on glycolytic metabolism. During exercise training adaptations towards the type I phenotype, the plantaris will have to activate some genes and silence others, such as fast-twitch genes. We hypothesize that mono-ubiquitinated H3K27me3 would show increased protein levels in the plantaris trained mice in order to ease activation of genes that support the type I phenotype.

We used an antibody that detects ubiquitination of H3 on the residue Lysine 23 in the gastrocnemius muscle. There was no significant difference in the levels of ubiquitinated H3 between sedentary and trained mice. Although there was no change in the ubiquitinated form of this particular lysine residue, this does not rule out the potential for ubiquitination on other lysine residues that may be affected by training.

In the short term training model, we observed results consistent with long term training. All analyses were done on the plantaris muscle. Our previous work on the same samples has shown that Mdm2 protein levels increase during short term training (Slopack et al., 2014). Therefore, Mdm2 was not investigated in this study. We found that EZH2 protein levels significantly increased within five days of training in mice. We also measured protein levels of phosphorylated EZH2, which revealed a significant decrease during training. Previous studies have shown several functions of EZH2 phosphorylation, notably that phosphorylation regulates EZH2 stability (Wei et al., 2011; Wu and Zhang, 2011). AMPK is known to phosphorylate EZH2 on Thr311 to suppress its methyltransferase activity (Wan et al., 2018). Recall that during exercise, AMPK acts rapidly

in response to imbalances in energy levels (Kjøbsted et al., 2018). This suggests that early bouts of exercise induces phosphorylation that has an inhibitory effect. Additionally, we have previously shown that a bout of exercise activates Mdm2 through phosphorylation on Ser166. These activations may tend toward a reduction of EZH2 function through phosphorylation and activation of Mdm2.

To understand the mechanism of changes in Mdm2, EZH2, and H3K27me3, we differentiated C2C12 myoblasts into myotubes over 5 days. We found that Mdm2 protein levels increased during differentiation. This was not surprising, as C2C12 cells adopt a more oxidative phenotype during differentiation and higher levels of Mdm2 are reported in more oxidative muscles, such as the soleus (Roudier et al., 2012; Wagatsuma and Sakuma, 2013). The increase in Mdm2 protein levels during C2C12 differentiation can be supported by other results in the present study. We have shown that Mdm2 protein levels were higher in the oxidative soleus muscle compared to the gastrocnemius and the plantaris. Additionally, long term endurance training, which leads to muscle cells adopting a more oxidative phenotype, caused an increase in Mdm2 protein levels in the gastrocnemius. Lastly, we know that angiogenesis occurred due to training, as the C:F ratio increased in the EDL muscle. Although we were not able to measure Mdm2 protein levels in the EDL, it is likely that this muscle would reveal higher levels of Mdm2 in trained mice compared to sedentary mice.

Our data has revealed a negative correlation between Mdm2 and EZH2 protein levels during C2C12 differentiation. EZH2 protein levels were depleted during C2C12 differentiation, which supports data from previous studies (Caretti et al., 2004). This suggests that higher levels of gene expression occur during myogenesis. When we probed for EZH2 in the C2C12 cells, we unexpectedly observed another upper band. The molecular weight of EZH2 is 98 kDa. The gel

electrophoresis was done on a 10% gel, therefore it was difficult to determine the approximate size of the band, since it appeared between the 100 and 150 kDa size markers which are close together. Visibly, we noticed that the upper band behaved in the opposite way compared to standard EZH2. Upon quantification, we confirmed that during C2C12 differentiation, the protein levels of this upper band significantly increased. We hypothesize that the upper band may be the result of PTMs on EZH2.

Interestingly, the upper band of EZH2 can also be seen in other cell types, such as HDMECs and SMECs. These can be found in supplemental figures. We were also able to detect the upper band of EZH2 in gastrocnemius of the long term trained mice, however, the band was not prominent enough for quantification. The upper band of EZH2 has not been investigated in other studies. Since the protein levels of the upper band are impacted during C2C12 differentiation, it is worth studying this band further. It would be valuable to find the molecular weight of the upper band, as well as learn what PTMs are present. Based on our current knowledge, we hypothesize that the upper band may be EZH2 with multiple mono-ubiquitinations, rather than polyubiquitination. This is because polyubiquitination marks proteins for degradation, therefore, they likely would not be highly detectable by experiments such as western blotting unless the proteasome activity is inhibited (e.g. use of MG132 inhibitor). We know that Mdm2 is an E3 ubiquitin ligase, and binds to EZH2 to place the trimethylation onto H3K27. Once the silencing marks have been placed, and EZH2 is no longer necessary, Mdm2 may mono-ubiquitinate EZH2 as a way to prime EZH2 for polyubiquitination, a post-translational modification that triggers proteasomal degradation. The regulation of EZH2 by Mdm2 may be different depending on the muscle type, since the relative stoichiometry differs from muscle to muscle. Further support for

this hypothesis comes from studies that have shown that EZH2 can be regulated by ubiquitination and by Mdm2 (Kuser-Abali et al., 2018; Wang et al., 2017).

We observed that during C2C12 differentiation, there is a highly variable, however significant increase in standard H3K27me3 at 17 kDa. This variability may be explained by the protein extraction of the C2C12 cells. It seems that some samples underwent more efficient histone extraction. This can be illustrated by protein levels of H3. H3, which is usually highly abundant in all samples and can be used as a loading control, was also variable. This is indicative of inefficient histone extraction from C2C12 cells. Additionally, the differentiation process has been shown to change chromatin compaction (Chen and Dent, 2014). Therefore, the variance in H3 levels may also be related to this physiological process. Overall, this data shows that gene silencing increased, at least up until day 2, of C2C12 differentiation. The lack of increase in the silencing mark after this time point matches the decrease in EZH2 protein levels observed during differentiation.

In addition, we observed that the protein levels of the putative mono-ubiquitinated H3K27me3 at 25 kDa steadily increased during C2C12 cell differentiation. This observation fits with the idea that higher levels of gene expression occur during C2C12 differentiation and that poised genes need to be reactivated. Mono-ubiquitination of H3K27me3 in this context would facilitate the acetylation of H3 and in turn allow for higher levels of gene expression. If this is the case, this observation also supports the decrease in EZH2, as less silencing is required and more activation is required during the process of myogenesis.

Lastly, we found that H3K27me3 and ubiquitin were bound after performing immunoprecipitation. This provides further evidence that H3K27me3 at 25 kDa may be mono-ubiquitinated. The shift in molecular weight that we reported here is similar to the observation made by Zhang et al. In human embryonic kidney cells and in human hepatocarcinoma cells

HEK297 and HepG3 cells, Zhang and colleagues observed that mono-ubiquitination of H3 on lysine reveals a band at 25 kDa (Zhang et al., 2017). In response to glucose stimulation, the E3 ubiquitin ligase Nedd4 catalyzes the mono-ubiquitination of H3 on lysine 23, 36, and 37. If the primary antibody is truly specifically detecting H3K27me3, our data might suggest that tri-methylated form of H3 on lysine 27 could be mono-ubiquitinated. However, it remains uncertain which E3 ligase could induce this ubiquitination.

Conclusion

Our study has shown that muscles expressing different phenotypic properties show a different relative abundance of the silencing mark H3K27me3. Long and short term training may also affect the presence of silencing marks. Our study has provided evidence that the abundance Mdm2 and EZH2 increase in response to different training exposures. The changes in the silencing marks in relation to muscle phenotype and endurance training point us in the right direction toward answering our hypothesis. More research is needed to fully elucidate Mdm2 and EZH2 interactions, and the role of gene silencing in these processes. Lastly, we have found some preliminary evidence to show that ubiquitination may be involved in maintaining muscle phenotype and during exercise training adaptations. Further studies will also help delineate whether the shift 25 kDa band detected by the H3K27me3 antibody is an epigenetic mark of interest for the plasticity of skeletal muscle phenotype.

Limitations and Future Directions

In the earlier stages of our study, we had not investigated the 25 kDa band of H3K27me3. This includes the experimental analyses for the short term trained mice and for both the plantaris and soleus muscles in the long term trained mice. As we progressed, we decided that the 25 kDa band was important for us to study. Therefore, it was included in most of our other later analyses. However, this study would greatly benefit from having these data.

Many experiments were completed and interpreted with the idea that the 25 kDa band may be due to a mono-ubiquitination of H3. However, we still have to investigate the specificity of this band and to examine what it is with further experiments. To determine if this is a post-translationally modified form of H3, we performed immunoprecipitation on a pool C2C12 extract from the time course differentiation. The pooled extract included both differentiated myoblasts (day 1-5) and undifferentiated myoblasts. This was the first immunoprecipitation that had been done for this study and it primarily served the purpose of testing our capacity to measure H3K27me3 using this experimental procedure. We will perform this experiment again in the future to ensure that the results that we have observed are accurate. We will also test other primary antibodies for H3K27me3 to assess whether the upper band can also be detected by other antibodies. However, since we were able to detect the upper band using the H3 antibody, this supports the idea that the upper band is a form of H3.

The data in this study are descriptive and we cannot conclude why or how changes occur. Mechanistic studies using inhibitors need to be completed in order to paint a full picture of the role of silencing marks in the context of muscle phenotype.

All animal studies in this project analyzed whole muscle homogenates. Whole muscle homogenates include all cell types found in skeletal muscle including endothelial, satellite,

fibroblasts, etc. These analyses can only tell us global levels of proteins. They cannot provide information about where exactly the proteins are located in the muscle. Similarly, here we have measured global levels of H3K27me3. However, the distribution of these silencing marks could change depending on where they exist on genes, without seeing any changes in overall levels of H3K27me3.

Due to the COVID-19 pandemic, some of the later experiments could not be completed. The missing data would certainly help complete the full picture of the epigenetic regulation that occurs to maintain muscle phenotype and as a result of training.

Planned experiments

Is histone 3 ubiquitinated in the gastrocnemius muscle of trained mice?

Since the 25 kDa band of H3K27me3 also remains unknown, my next step is to investigate what PTM may be causing the shift in weight. Using the co-immunoprecipitation protocol, we can isolate histone 3 from the gastrocnemius extract from trained mice and then probe for ubiquitin by western blotting. This will allow us to determine whether or not the band we are seeing at 25 kDa band is mono-ubiquitin, as hypothesized. Our next step will be to treat the gastrocnemius muscle extract with deubiquitinase. This will reveal whether the upper band is truly due to a mono-ubiquitin PTM.

Is H3K27me3 bound to ubiquitin in C2C12 cells? What are the mechanisms for H3 ubiquitination?

To further understand the mechanisms of potential H3 ubiquitination, we will use C2C12 cells as an in-vitro model. This model will be utilized to modulate Mdm2 and EZH2 activity with inhibitors. C2C12 cells will undergo differentiation and then electrical pulse stimulation. Since H3K27me3 at the 25 kDa band is most abundant in C2C12 cells on day 5 of differentiation, we will study the H3 ubiquitination in differentiated C2C12 cells by immunoprecipitation.

Are Mdm2 and EZH2 bound in skeletal muscle and C2C12 cells? Is Mdm2 required for C2C12 differentiation?

Immunoprecipitation of Mdm2 and blot for EZH2 in the C2C12 cells, as well as in the gastrocnemius protein extracts will help us determine if Mdm2 and EZH2 are bound in both these tissues. This will ultimately lead us to elucidate if the interaction between Mdm2 and EZH2 plays a role in exercise training adaptation and the maintenance of muscle phenotype.

Is Mdm2 required for C2C12 differentiation?

Treating C2C12 cell culture undergoing time course differentiation with Mdm2 inhibitor nutlin-3 can help us determine if Mdm2 is required for C2C12 differentiation.

Are the shifts in molecular weights observed for EZH2 and H3K27me3 due to a ubiquitin PTM?

We will use Immunoprecipitation-Mass spectrometry (IP-MS) as an analysis technique to determine the exact molecular weight of the upper band of EZH2 and the upper band of H3K27me3. IP-MS will also help us determine all the PTMs present on H3 or EZH2. This technique can be done on an SDS gel after proteins issued from IP have been discriminated by electrophoresis. We will use the EZH2 and H3K27me3-specific antibodies to perform the IP.

Measuring H3K27me3 presence on genes of interest

It would be interesting to investigate whether there are silencing marks on genes coding previously reported to be regulated by Mdm2 and EZH2 through H3K27me3. Wienken and colleagues showed Mdm2 and EZH2 could determine the fate of stem cells by regulating genes, such as Hox genes, which are important for differentiation and potentially muscle differentiation (Schwörer et al., 2016; Yamamoto and Kuroiwa, 2003). However, this remains unstudied in C2C12 cells. Finally, it would be valuable to investigate the presence of silencing marks on genes related to the fast twitch phenotype in trained muscles compared to sedentary. We will do so by performing chromatin immunoprecipitation of the gastrocnemius muscle. Since changes in muscle phenotype coordinate with changes in fiber type and angiogenesis, it may also be valuable to study silencing marks on angiogenesis-related genes known to regulate Mdm2 and EZH2, such as Notch1 and KDR.

Supplemental Figures

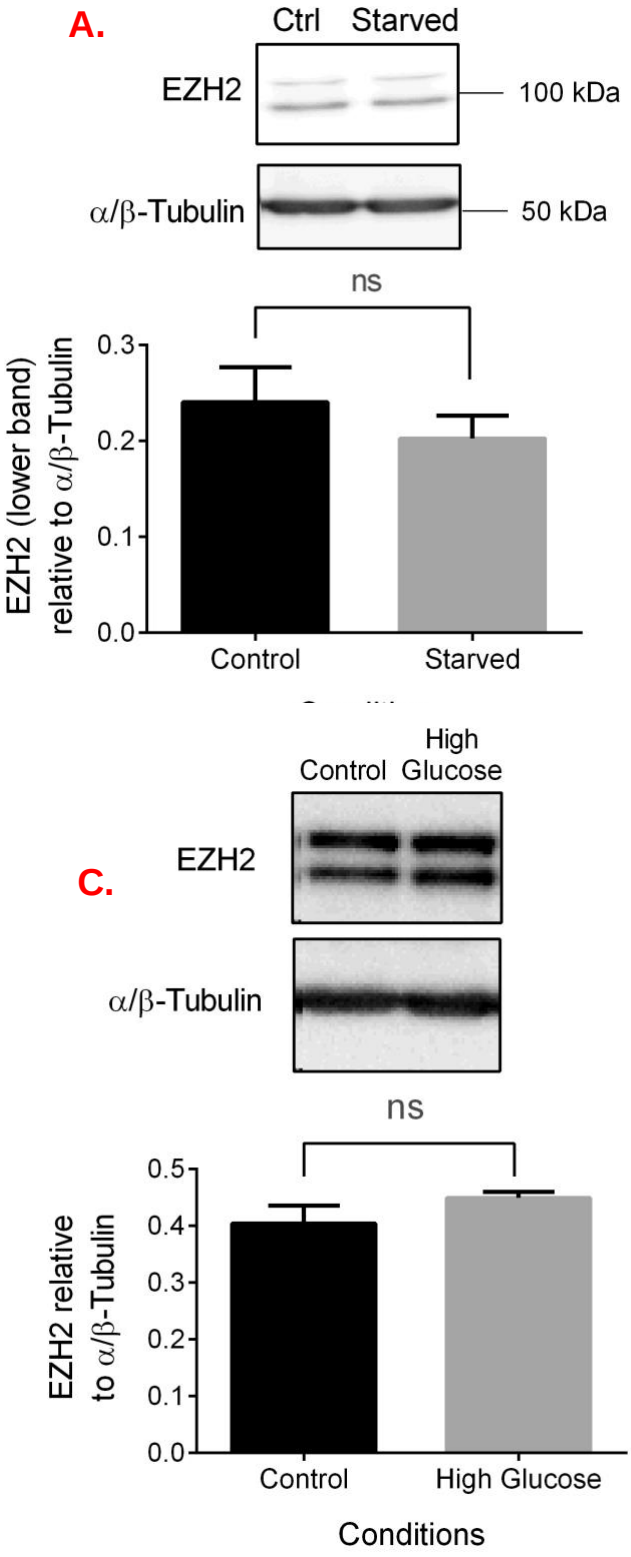


Figure 2.14. HDMECs and SMECs also show an upper band for EZH2.
The samples analyzed were control and treated HDMECs or SMECs. **A.** shows EZH2 protein levels relative to α/β-Tubulin in HDMECs under controlled or started conditions. **B.** shows EZH2 protein levels relative to α/β-Tubulin in HDMECs under control or VEGF treatment. **C.** shows EZH2 protein levels relative to α/β-Tubulin in SMECs under control or high glucose treatment. The molecular weight represents the nearest marker to the molecular weight of the protein. Significant effect of differentiation ($p<0.05$) (Student's T-test) *=$p<0.05$. n=6 per group. Data presented as: mean ± SEM.

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