DOES EPIGENETIC SILENCING AFFECT SKELETAL MUSCLE ANGIOGENESIS?

SOKAINA AKHTAR

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

GRADUATE PROGRAM IN KINESIOLOGY AND HEALTH SCIENCE

YORK UNIVERSITY

TORONTO, ONTARIO

DECEMBER, 2022

Abstract

Background: The skeletal muscle is a highly plastic tissue that is able to adapt to changes from different stressors such as exercise. Repeated bouts of physical activity cause changes in the skeletal muscles' metabolic and structural properties. Angiogenesis is the formation of new capillaries from existing vessels; this adaptation allows for better blood flow to the working skeletal muscles. Multiple molecular changes work together during exercise to allow for skeletal muscle adaptations, which requires change in gene expression. Epigenetic modifications are reversible changes to the chromatin structure, allowing for certain genes to be activated and others to be silenced. MDM2 is an E3 ubiquitin ligase known for its proangiogenic functions and can play a role in epigenetic modifications. MDM2 increases in response to endurance training and is crucial for skeletal muscle angiogenesis. EZH2 also shares epigenetic functions as a histone methyltransferase within the transcriptional repressor complex PRC2 that methylates H3K27. Interaction between MDM2 and EZH2 further supports silencing of certain angiogenic genes through the tri-methylation of Histone 3 (H3K27me3).

Rationale: There is a lack of research on how histone modifications, specifically gene silencing ones, regulate skeletal muscle adaptations through angiogenesis in response to training. My thesis aims to research how histone silencing marks regulate skeletal muscle angiogenesis in the context of exercise.

Results: H3K27me3 protein global abundance at a previously unreported molecular weight, increases with endurance training and decreases with an acute bout of exercise in 14 days trained C57BL6 female mice. MDM2 decreases 3 hours after an acute bout of exercise in 14 days trained C57BL6 female mice. Angiogenic gene *Kdr* known to be regulated by MDM2 and EZH2, decreases in mRNA expression after 9 weeks of endurance training. With EZH2 inhibition, *Kdr* mRNA increases in mSMECs. Immunoprecipitation reveals that unreported molecular weight of H3K27me3 at the 25 kDA band could potentially be a ubiquitinated form of H3. Chromatin immunoprecipitation suggests there is a tendency for increased H3K27me3 silencing marks on the KDR gene in 9 weeks trained gastrocnemius muscle.

Conclusion: Exercise protocols affect the histone modifications and histone modifiers in the skeletal muscle.

Dedication

This thesis is dedicated to my family for being extremely supportive and patient with me throughout my Masters studies; I would not be where I am today without you all. I would like to specially thank my mom and my sister, you both inspired and motivated me to achieve my best.

Acknowledgements

I would first like to express my utmost gratitude to my supervisor Dr. Emilie Roudier for endlessly supporting me throughout my Masters studies; I have truly learnt many valuable skills through your guidance and care. Without your knowledge, patience, support, and encouragement, this work would not be possible.

I would like to thank my supervisory committee member, Dr. Olivier Birot, for his knowledgeable insight and continuous support and feedback for my project. I truly appreciate it.

I would also like to thank Dr. Elizabeth Clare for taking the time to evaluate my thesis.

I would like to specially thank my lab members Brian and Monica for training and supporting me throughout my project. I am also grateful for my current and past lab members: Brian, Monica, Pierre, Khalil, and Dayna for all their help and in making research an enjoyable experience.

CONTENTS

List of figures

List of figures in chapter 1: introduction:

List of figures in chapter 2: study design:

List of figures in chapter 4: results:

List of figures in chapter 5: Discussion:

List of figures in chapter 6: Supplemental Data:

Abbreviations

ADP – Adenosine diphosphate

ATP – Adenosine triphosphate

BCA – Bicinchoninic acid

CD36 - Cluster of differentiation 36

CD31 – Cluster of differentiation 31

COXIV – Cytochrome C Oxidase Subunit 4

CFPE – Capillary to fiber perimeter exchange index

CO_2– Carbon dioxide

ChIP – Chromatin immuno- precipitation

CpG – Cytosine guanosine dinucleotide 5’—C—phosphate—G—3'

DNMTs- DNA methyltransferase

DNB – Denatured buffer

DMSO – Dimethyl sulfoxide

DLL4 – Delta like canonical Notch ligand 4

ddH_2O – Double distilled water

EC – Endothelial cells

EDTA – Ethylenediaminetetraacetic acid

ERRα – Estrogen related receptor alpha

EZH2 – Enhancer of zeste homolog 2

FFA – Free fatty acids

FATP1 – Fatty acid transport protein 1

Flk -1 – Fetal liver kinase 1 (KDR)

Flt-1 – FMS - like tyrosine kinase receptor 1

FABP – Fatty acid binding protein

GSK343 – SAM – competitive inhibitor of the histone lysine methyltransferase EZH2

GLUT 1 – Glucose transporter 1

GLUT 4 – Glucose transporter 4

HPRT – Hypoxanthine guanine phosphoribosyl transferase

HRP – Horse Radish Peroxidase

HIF-1α – Hypoxia Inducible Factor 1 alpha

IgG – Immuno- globin

IP – Immuno- precipitation

KDR (VEGFR2) – Kinase Insert Domain Receptor (VEGF receptor 2)

MEFs – Mouse embryonic fibroblasts

mSMECs – Mouse skeletal microvascular endothelial cells

MDM2 – Murine Double Minute 2

mRNA – Messenger RNA

miRNA – Micro RNA

MVU – Microvascular unit

NEDD4 – Neural precursor cell expressed developmentally down regulated protein 4

NO – Nitric oxide

NOTCH – Neurogenic locus notch homolog protein 1

NaF – Sodium fluoride

Na_3VO_4 – Sodium orthovanadate

PcG – Polycomb group proteins

PlGF - Placental growth factor

p-EZH2 – Phosphorylated Enhancer of Zeste Homolog 2

p-MDM2 – Phosphorylated Murine Double Minute 2

P53 – Tumor protein 53

PMSF – Phenylmethylsulfonyl fluoride

PECAM (CD31) – Platelet endothelial cell adhesion molecule-1

PFK – Phosphofructokinase

PPARs – Peroxisome proliferator- activated receptors

PGC-1α – Peroxisome proliferator-activated receptor-γ Coactivator-1α

PO_2 – Partial pressure in oxygen

PRC1 – Polycomb repressive complex 1

PRC2 – Polycomb repressive complex 2

PBS – Phosphate buffered saline

RIPA – Radio Immuno-Precipitation Assay

RING – Really interesting new gene

RNA – Ribose Nucleic Acid

SDS – Sodium dodecyl sulfate

SDH - Succinate dehydrogenase

S-2HG – S-2 hydroxyglutarate

TSS – Transcription start site

TSP-1 – Thrombospondin 1

VEGF-A – Vascular endothelial growth factor

VO_2 – Maximal oxygen uptake

VEGFR2 – Vascular endothelial growth factor receptor 2

Chapter 1: Introduction

1.1. Context of my research: the health benefits of exercise

Exercise is a well-known beneficial intervention for the prevention of many diseases such as cardiovascular diseases, hypertension, type 2 diabetes, or obesity (Adams et al., 2017) whereas low levels of physical activity increase the prevalence of these chronic diseases (as reviewed by Lavie et al., 2015). The beneficial impact of exercise is partly driven through the maintenance of functional blood vessels so they can supply healthy skeletal muscles.

Research by Cornelissen et al in 2010 showed that when sedentary men and women at the age of 55 years participated in endurance training, they could significantly decrease their systolic blood pressure and resting heart rate (Cornelissen et al., 2010). A study done by Vaitkevicius on arterial stiffness, age, and VO_2 max reported that senior trained individuals exhibited lower aortic pulse wave velocity (an indicator of aortic stiffness) than the sedentary senior individuals of the same age (Vaitkevicius, 1993). Endothelial cells cover the inner most layer of a tissue called the endothelium, which is central to the vascular function. The vascular endothelial function is usually measured through dilation of forearm blood flow by an external stimulus that induces the synthesis of nitric oxide (NO) (Seals, 2014). Acetylcholine is one stimulus that is used for this measurement and with regards to exercise, trained older subjects were seen to have the same forearm blood flow responses when stimulated with acetylcholine as younger subjects, suggesting that exercise helps maintain vascular endothelial function with aging (as reviewed by Seals, 2014).

With respect to skeletal muscle, there are several adaptations that occur in response to exercise. Research has shown that exercise increases mitochondrial content, fat oxidation, and capillary density (see Figure 1.1). In a group of rats, gastrocnemius and soleus muscles of endurance trained rats exhibited increases in cytochrome oxidase and succinate oxidase content (mitochondrial enzymes) compared to sedentary rats, suggesting that mitochondrial content has increased due to training (Holloszy, 1967). In human participants, with exercise performed at the same relative intensity, endurance trained individuals

had higher fat oxidation than sedentary individuals, due to increased rate of fatty acid entry in the mitochondria (Sidossis et al., 1998). The microvasculature of skeletal muscle is crucial in supporting nutrient supply, waste removal, and gas exchange (Hendrickse & Degens, 2019). The capillaries form most of the skeletal muscle microvasculature, as described further in paragraph 1.2., "skeletal muscle microvasculature". An increase in capillary density (number of capillaries per cross- sectional area of muscle; see Figure 1.3B) is reported after exercise training. This improvement in muscle capillarization ensures that the blood supply meets the metabolic demands from exercising myofibers. Four days of electro-stimulation (8 hours per day) of rat muscles led to significant increases in the number of capillaries that supply blood to a muscle fiber (as measured as capillary to fiber ratio; see Figure 1.3B) (Hansen-smith et al., 1996). With an eight-week endurance training protocol, quadricep muscles of males showed an increase in capillary to fiber ratio and capillary density (Andersen & Henriksson, 1977).

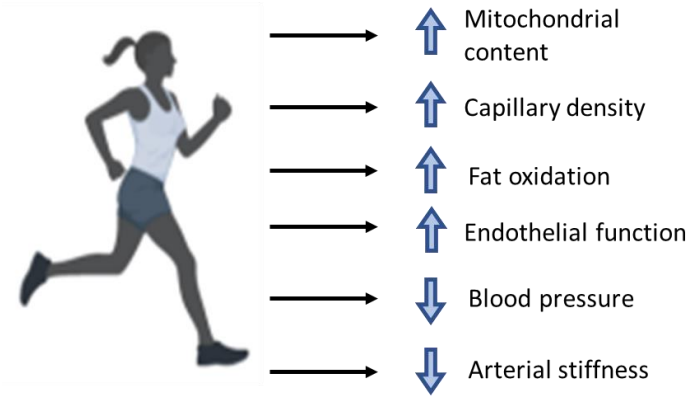


Figure 1.1: Beneficial impact of exercise training on the whole-body metabolism, skeletal muscle, and the vascular system. Exercise training supports an increase in the mitochondrial content in the skeletal muscle, as well as a greater oxidative metabolism (fat oxidation) in the muscle tissue, and in the whole body. Exercise training promotes vascular adaptations in the microvasculature (e.g., greater capillary density in the skeletal muscle), in the arteries (e.g., lower arterial stiffness), and enhance the endothelial functions to support a better regulation of blood pressure. Together, these changes enhance the cardiovascular and metabolic functions, supporting better health. Image created on Biorender.

Research has proven on multiple occasions how crucial exercise is for maintaining the health of the human body and preventing diseases. The focus of my thesis will be solely on the exercise adaptations in the skeletal muscle, and more particularly in the context of capillarization and angiogenesis.

1.1.1. Exercise and the skeletal muscle

The skeletal muscle is a highly plastic tissue that makes up about 40% of our body's weight (Frontera & Ochala, 2015; Mukund & Subramaniam, 2020). The functions of the skeletal muscles are vast. From a biomechanical perspective, skeletal muscles enable postural support as well as force and movement generation (Frontera & Ochala, 2015). In terms of metabolic properties, the skeletal muscle functions include regulating body temperature, contributing to basal energy metabolism, and the intake of oxygen and fuel during exercise (Frontera & Ochala, 2015). With movement, for example an acute bout of exercise, skeletal muscles need energy (ATP) to contract (Frontera & Ochala, 2015), with the smallest unit of contraction in the muscle being the motor unit (Saltin et al., 1977).

The mechanism behind how skeletal muscle contraction occurs was highlighted by Huxley's sliding filament theory. Skeletal muscle fibers contain contractile protein known as myosin and actin filaments, which are arranged in a repeating pattern known as sarcomeres (Brooks, 2003). Briefly, during a contraction, the cross-bridge cycle (see Figure 1.2) is activated in which ATP binds to myosin. Actin and myosin then detach, hydrolysis of ATP occurs; which results in free energy, ADP, and a phosphate group (P_i). ADP and P_i remain attached to myosin as myosin head attaches to a new actin filament. Once ADP and P_i are released from myosin, myosin and the new actin filament resume the tight attachment (Brooks, 2003; Frontera & Ochala, 2015). This results in a shortened sarcomere and ultimately the contraction of the muscle.

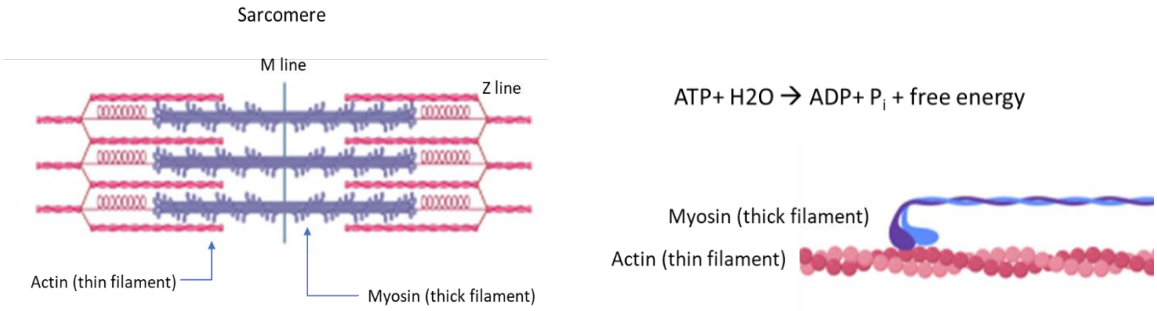


Figure 1.2: Diagram representing the crossbridge cycle in skeletal muscle. Left image depicts the whole sarcomere structure in the myofibers. Right image shows close up view of myosin head interacting with the actin filament. Crossbridge activation is due to ATP energy in skeletal muscle (see chemical reaction above). Image created on Biorender.

1.1.2. Types of muscle fibers

The skeletal muscle is composed of different fibers that vary in properties. Muscle fiber types have been classified by different properties such as the colour of muscle fiber, shortening speed during a single twitch, and metabolic or enzymatic pathways that muscle fibers predominantly have (Frontera & Ochala, 2015). The main fiber types in skeletal muscles are type 1, type 2A, and type 2X (Scott et al., 2001). Myoglobin is a protein expressed in muscles that is rich in iron and deals with oxygen storage and diffusion; type 1 fibers are red in colour due to their high myoglobin and capillary content, while type 2 fibers are white in colour (Scott et al., 2001; Schiaffino & Reggiani, 2011). Type 2 fibers are also observed to have more glycolytic enzymes while type 1 fibers contained more oxidative (Scott et al., 2001; Schiaffino & Reggiani, 2011). Myosin ATP hydrolysis rate was observed to be 2-3 times greater in fast twitch fibers compared to slow twitch muscle fibers (as reviewed by Scott et al., 2001). Therefore, the classification of muscle fibers is as followed: type 1 fibers are red, slow, and oxidative, type 2A are white, fast-twitch, oxidative-glycolytic, and type 2X are white, fast-twitch, glycolytic (Schiaffino & Reggiani, 2011). The metabolic properties of different human fibers have been studied extensively; it was observed that the triglyceride and succinate dehydrogenase (SDH) activity was higher in type 1 than type 2 fibers while ATPase activity levels and the glycolytic enzyme phosphofructokinase (PFK) was higher in type 2 fibers

(Essen et al., 1975). In response to physical activity in humans, all fiber types have been seen to increase in the size of the fiber (Saltin et al., 1977). Physical inactivity led to decreased SDH activity and only the type 1 fibers of sedentary individuals exhibited the highest oxidative capacity (Saltin et al., 1977). With training, SDH activity was seen to be as high in type 2 as in type 1 (Saltin et al., 1977). This suggests that with training, type 2 fibers can increase their oxidative capacities.

In a study observing fiber composition in 6 men and women measured in muscle biopsies of the triceps, vastus lateralis, and soleus muscles, suggested that fiber type differences in capillary supply in humans are less distinct than in other mammals (Sjøgaard, 1982). The number of capillaries per fiber were higher in the soleus which had the highest slow twitch fiber composition as well, compared to the vastus lateralis and the triceps muscles in humans; yet, the capillary density was similar between those muscles (capillary density was measured capillaries per mm^2 of muscle tissue) (Sjøgaard, 1982). In muscle biopsies of soleus and the lateral head of the gastrocnemius in men, both muscles were seen to have a mix of fiber types, however the soleus had higher slow twitch fibers compared to the gastrocnemius (Andersen & Kroese, 1978). With respect to capillary supply, even though the capillary density was observed to be higher in the gastrocnemius compared to the soleus, the capillaries were seen to be arranged differently in those muscles, with the gastrocnemius capillaries arranged parallel to the muscle fiber and the soleus capillaries in a more branching arrangement (Andersen & Kroese, 1978). Therefore, a true comparison in capillary supply between the gastrocnemius and soleus muscle of men were not stated in the previous study.

The capillary supply in the skeletal muscle is crucial as it matches the metabolic needs of the muscle tissues, being low in demand during rest, and high during exercise (Wagenmakers et al., 2016) and has been observed to adapt in the context of exercise and training.

1.2. Skeletal muscle microvasculature

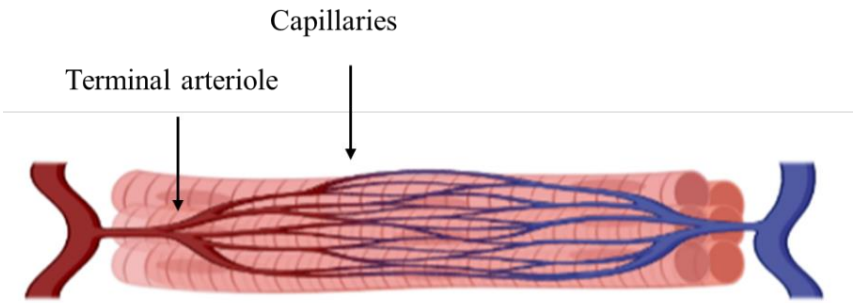
The capillary network in the skeletal muscle is dense and delivers oxygen and nutrients to its tissue and removes waste and heat from it (Hendrickse & Degens, 2019). The skeletal muscle microvasculature consists of arterioles, venules, and capillaries. The arteriolar network (paired with its venous network) branches from the artery that enters the muscle (Murrant et al., 2021).

Briefly, the structure of the skeletal muscle can be described as striated and are made up of muscle fibers (myofibers) arranged by connective tissue with each myofiber containing many myofibrils (Mukund & Subramaniam, 2020; Frontera & Ochala, 2015). Skeletal muscle fibers are multinucleated due to the fusion of many myoblasts (Okazaki & Holtzer, 1966), which allows for the protein in that section of muscle fiber to be controlled by that specific nucleus (Frontera & Ochala, 2015). The muscle stem cells, i.e., satellite cells, lie along the myofibers to support muscle regeneration and post-natal growth (Latroche et al., 2015). The relative size of the muscle fiber varies depending on the type of muscle. The range of length for a human myofiber is observed to be between 4-40 cm and the diameter of the capillaries is reported to be 6-10 μm (as reviewed by Wagenmakers et al., 2016). The microvasculature surrounds these myofibers, with the capillaries elongated along the myofibers being in close contact with muscle cells' nuclei, including myofibers and satellite cells, to create a microenvironment that would support muscle function and health.

1.2.1. The anatomy of a microvascular unit

The arterioles in the skeletal muscle further branch into daughter arterioles ending at the terminal arteriole. The microvascular unit (MVU) is the smallest unit that regulates blood flow and refers to the network of capillaries that are perfused through a terminal arteriole (see Figure 1.3) in the skeletal muscle (Segal, 2005; Murrant et al., 2021). The MVU runs along the myofibers with the length around 500-1700 μm depending on the skeletal muscle type (Murrant et al., 2021). Each MVU consists of 5-10 capillaries that are perfusing between 3-4 myofibers, with one MVU perfusing a small area on multiple myofibers, and each myofiber being perfused by multiple different capillary units from different terminal arterioles (Latroche et al., 2015; Murrant et al., 2021).

A



B

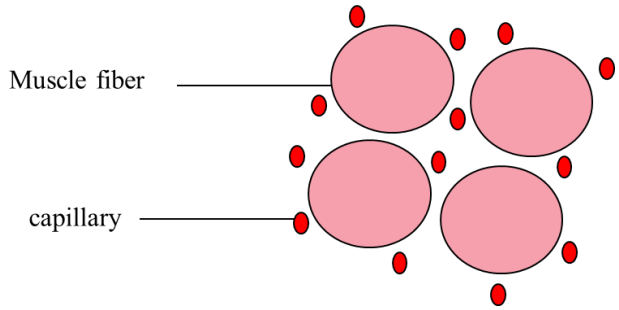


Figure 1.3: Transversal and cross-section views of the skeletal muscle microvascular unit. A) Schematic representation of at transversal view of a microvascular unit in the skeletal muscle. **B)** Cross-sectional view of a skeletal muscle showing capillaries surrounding the myofibers. Assessment of the capillarization can be done by measuring the capillary density (CD; number of capillaries per cross- sectional area of muscle, here CD is equal to 13) and by measuring the capillary to fiber ratio (C:F ratio; number of capillaries in a field of view divided by the number of myofibers in the same field of view, here C:F ratio is equal to 13/4). Image created on Biorender.

1.2.2. Skeletal muscle capillaries: structure and function

The structure of a capillary (see Figure 1.4) consists of a single layer of endothelial cell lining (Hudlicka, 2011). The surface of capillaries is partially covered with pericytes, which provide structural support and help in endothelial cell proliferation and remodeling of the capillaries into arterioles (Hudlicka, 2011).

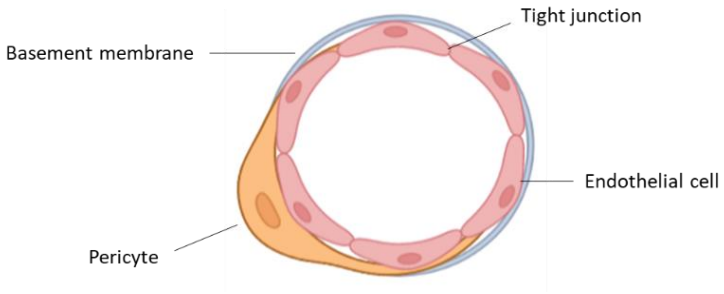


Figure 1.4: Schematic representation of a capillary cross section in the skeletal muscle. ECs are covered by pericytes and have tight gap junctions in between each other. Image created on Biorender.

In the region where the terminal arteriole transitions into a capillary section, the endothelial cells that form the capillary are connected to smooth muscle cells in the gap junctions between arteriolar and capillary endothelial cells (Hudlicka, 2011). The capillary structure helps in oxygen and blood flow delivery to the skeletal muscle fibers. As explained above, a skeletal muscle fiber receives blood supply from multiple capillary units due to the length of the muscle fiber being longer than the capillary unit. The perfusion of these capillaries occurs from the terminal arteriole since capillaries do not change their diameter; when vasodilation occurs, the terminal arteriole is giving rise in perfusion to the capillaries, increasing the blood flow (Parise et al., 2020). Since the gap junctions in the capillary are connected to the smooth muscle cells (as outlined above), signals can be transduced from the capillary to the adjacent terminal arteriole, a term known as a conducted response. A conducted response is essentially capillary communication, where a vasodilatory signal can spread in the smooth muscle cells due to endothelial cell hyperpolarization (Parise et al., 2020). This can occur for example during muscle contraction where the contraction of the myofiber stimulates the surrounding capillaries through hyperpolarization of the endothelial cells, sending the vasodilatory signals to the terminal arteriole via smooth muscle cells for allowing dilation and increase in blood flow (Parise et al., 2020). Conducted responses in capillaries can occur from muscle contraction, nitric oxide (NO), acetylcholine, adenosine, and potassium (Ballard, 2014; Parise et al., 2020). This allows for capillaries to aid in controlling blood flow to meet metabolic demands during exercise.

By providing nutrient and oxygen, the flow of blood in the muscle supports energy or ATP production within the skeletal muscles. A process mainly achieved through the utilization of glucose or free fatty acids. The uptake of free fatty acids (FFA) supports ATP production through the process of beta oxidation (Mazibuko-Mbeje et al., 2018). Fatty acid transporters such as cluster of differentiation 36 (CD36), fatty acid transport protein 1 (FATP1), and fatty acid binding protein (FABP) are all present on skeletal muscles (Mazibuko-Mbeje et al., 2018). Glucose uptake in the skeletal muscles occurs through GLUT 4 which trans-locates from the cytoplasm to the plasma membrane of the myocytes in response to insulin (Mazibuko-Mbeje et al., 2018). The uptake of the substrate glucose and FFAs are utilized to produce Acetyl – CoA which would then in turn aid in the production of ATP for energy (Mazibuko-Mbeje et al., 2018). This helps in energy production for contractile activity due to exercise. Exercise is not the only mechanism that can increase capillary blood flow in the skeletal muscle; insulin has been observed to as well (Wagenmakers et al., 2016). GLUT 1 transporters are located on the ECs, meaning that the skeletal muscle capillaries also will have GLUT 1 present (Wagenmakers et al., 2016). The increasing concentration of insulin in the interstitial fluid around the muscle fibers increases the glucose uptake in the muscle through the insulin signaling pathway which leads to the increase in the production of NO which then vasodilates the arterioles and increases the capillary blood flow (Wagenmakers et al., 2016).

1.2.3. Exercise and skeletal muscle capillarization

As highlighted earlier, during exercise the energy demand increases in the skeletal muscle. For example, with aerobic exercise the energy expenditure of skeletal muscles increases by 160-fold versus at rest, which trained athletes are seen to maintain for more than 2 hours post exercise (as reviewed by Wagenmakers et al., 2016). This is due to their increased mitochondrial and microvascular networks that match their energy demands compared to sedentary individuals (as reviewed by Wagenmakers et al., 2016). With this increase in energy demand, transportation of energy substrates such as oxygen, glucose, and fatty acids are sent to the muscles through the blood, making the capillary supply crucial to ensure that the demands are met (Wagenmakers et al., 2016). In fact, during contractile activity, the capillary distance from

the myofiber was observed to be at approximately 60 µm compared to around 200 µm when at rest (as reviewed in Segal, 2005). A reduced distance between the capillaries and the myofibers supports faster oxygen diffusion facilitating oxygen uptake by the myofibers.

With regards to exercise training, multiple studies showed that training helps in building a better skeletal muscle microvasculature. The increase observed in aerobic capacity in the lower limbs of post endurance trained 12 healthy older subjects were linked to the increase in capillary to fiber interface (measure of maximal O_2 flow in the fiber) and increased microvascular filtration capacity (Charles et al., 2006). With nine weeks of resistance training in older men, the fiber shape and the diffusion distance did not change after training; however, there was an increase in number of capillary contacts around the fiber and an increase in capillary to fiber perimeter exchange index (CFPE) which is the quotient of the capillary to fiber ratio (number of capillaries for a fiber) and the perimeter of the said fiber (Hepple et al., 1996). In terms of both resistance and aerobic training, 20 healthy older men were put in either nine weeks of lower body resistance training followed by nine weeks of aerobic training or in eighteen weeks of aerobic training; in both training methods, the number of capillaries per fiber perimeter length increased but capillary density (number of capillaries per cross- sectional area of muscle) only increased after aerobic training (Hepple et al., 1997). Both types of exercise training are seen to be beneficial for the microvasculature.

When capillarization and capillary supply of muscle fibers were compared between young and old subjects, the physically active older individuals showed no significant decrease in muscle fiber oxidative capacity (Barnouin et al., 2017). Older subjects did show type 2 fiber atrophy and capillary rarefaction; however, the fiber size and capillary supply relationship was maintained (Barnouin et al., 2017). When looking at how chronic diseases affect the microvasculature, a study done on muscle biopsies of chronic heart failure patients showed reduced capillary density and reduced peak oxygen consumption in comparison to the control subjects (Duscha et al., 1999). These studies suggest that exercise indeed improves the microvasculature and can maintain it throughout the aging process. In cases of chronic

diseases, the microvasculature is negatively affected which translates to impaired oxygen utilization for the vascular system.

When bouts of exercise are repeated over weeks in the context of training, the successive exposures to recurrent changes in muscle activity supports an increase in capillarity aiming to achieve a better fit between the blood supply and the metabolic demand through a process known as angiogenesis, which is the formation of new capillaries from pre-existing blood vessels (Adair & Montani, 2010). The section below will describe in more details the structural changes observed in the skeletal muscle MVU and the process of angiogenesis.

1.3. Angiogenesis: the process of capillary formation

Angiogenesis is a process that is tightly regulated by pro and anti-angiogenic factors, and takes place throughout different stages of life starting from embryonic growth (Adair & Montani, 2010; Egginton, 2011; Felmeden, 2003). It is rarely seen in adult tissues except in response to exercise, wound healing, in females' endometrium during the menstrual cycle, and during pathophysiological processes (Adair & Montani, 2010; Felmeden, 2003).

Angiogenesis can be divided into sprouting and splitting (intussusception) angiogenesis (Adair & Montani, 2010). Sprouting angiogenesis can add blood vessels to areas of tissue that are less vascularized by the degradation of capillary basement membrane, endothelial cell proliferation and migration, formation of tubes, vessel fusion, and the stabilization of pericytes (Adair & Montani, 2010). This process is activated in response to high concentration of pro-angiogenic signals (Adair & Montani, 2010; Gorski & Bock, 2019). Firstly, quiescent ECs become tip cells and send out thin long projections called filopodia (Adair & Montani, 2010; Gorski & Bock, 2019). The cells adjacent to the tip cells become stalk cells that proliferate to elongate the sprout from behind the tip cell and form the lumen (Adair & Montani, 2010; Gorski & Bock, 2019). Splitting angiogenesis occurs when a single vessel splits into two by the vessel wall extending into

the lumen (Adair & Montani, 2010). A hole is created in the contact area of the two opposing capillary walls shaping a vessel like structure (Gorski & Bock, 2019). Although this process is relatively faster compared to sprouting, as it does not require immediate EC proliferation or migration; it only creates new capillaries in areas of tissues that already exist (Adair & Montani, 2010; Gorski & Bock, 2019). Splitting angiogenesis is seen to take place prominently in the vascular growth of embryos, hence the faster process of capillary growth (Adair & Montani, 2010).

Sprouting angiogenesis (see Figure 1.5) is the most common form of angiogenesis that has been studied (Egginton, 2008). The mechanisms behind sprouting angiogenesis occur in response to high levels of pro-angiogenic molecules. The vascular endothelial growth factor- A (VEGF-A) is one of the most studied pro-angiogenic molecules in the context of skeletal muscle angiogenesis. ECs express levels of the tyrosine kinase receptor 1 (Flt-1 or VEGFR1) and 2 (Flk-1 or VEGFR2, coded by the KDR gene) (Adair & Montani, 2010; Milkiewicz et al., 2003). The binding of VEGF-A onto the VEGFR2 in these ECs allow them to migrate and become tip cells, which are cells leading the migration front of the newly formed sprout (Adair & Montani, 2010). Additionally, VEGF-A induces the expression of delta-like 4 (DLL4) which is a notch ligand, in tip endothelial cells. Binding of DLL4 to its receptor on adjacent ECs in turn activates Notch signalling, forcing these cells to become stalk endothelial cells (Kume, 2009). This down regulates the expression of VEGF-A receptors and reduces the sensitivity to VEGF-A stimulation, which allows for the prevention of excessive migration and proliferation (Kume, 2009; Siekmann & Lawson, 2007).

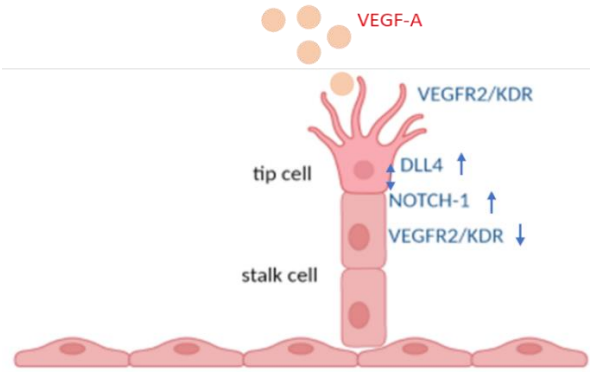


Figure 1.5: Diagram of sprouting angiogenesis in the endothelial cells. VEGF-A binds to receptor VEGFR2/KDR and increases DLL4 expression in the tip endothelial cells. DLL4 binds to NOTCH-1 ligands and increases the expression of NOTCH-1 in the stalk endothelial cells which then down regulates VEGFR2/KDR in the stalk cells to avoid excessive tip cells production and proliferation. Image created on Biorender.

1.3.1. Exercise induced skeletal muscle angiogenesis

The physiological environment is important in triggering angiogenesis and capillary growth. Skeletal muscle capillary growth is stimulated by mechanical influences, including shear stress induced by blood flow and stretch when myofibers contract (Hoier et al., 2012) as well as metabolic factors, i.e., changes in PO_2 and release of metabolites (e.g., lactate) mainly produced by the myofibers or resulting from the increase myofiber activity during contraction. The changes in muscle metabolism and oxygen utilization in the working muscle provides stimulus for capillary growth (Hoier et al., 2012). Capillary growth can also take place when a stimulus such as hypoxia is detected in the tissues which promotes the secretion of pro-angiogenic molecule vascular endothelial growth factor (VEGF-A) (Adair & Montani, 2010). While NO does provide a stimulus for capillary growth, it is not required; however, VEGF-A is needed for both splitting and sprouting angiogenesis to occur (Egginton, 2011). Adenosine, which is formed through skeletal muscle contractions, can also stimulate production of VEGF-A which would promote angiogenesis (Ballard, 2014; Hoier et al., 2010). When either 125 or 1250 µm of adenosine was administered in human skeletal muscles, the interstitial VEGF concentrations increased by 2.5 and 4-fold (Hoier et al., 2010). Stretch is also a mechanical stimulus for angiogenesis. In rats, EDL muscles were overloaded by stretch after the removal of the TA muscle (Egginton et al., 1998). After two weeks, the sarcomere length increased

from 2.4 to 2.9 µm and the capillary to fiber ratio increased by 33% (Egginton et al., 1998). After eight weeks there was a 60% increase in capillary to fiber ratio (Egginton et al., 1998). With respect to ischemia as a stimulus for angiogenesis, chronic ischemia (after 5 weeks) in the rats' EDL muscle by a single ligation showed a 20% increase in capillary to fiber ratio (Milkiewicz et al., 2003). With double ligation and intermittent electrical stimulation, ischaemic muscles showed a 22% increase in capillary to fiber ratio (Milkiewicz et al., 2003). After 2 weeks of ischemia by single ligation, EDL muscles showed 60% increase in VEGF-A protein levels and after 5 weeks VEGF-A protein levels increased by 80% (Milkiewicz et al., 2003). The VEGF-A receptor Flk-1 (VEGFR2) protein levels were doubled from basal level in muscles after two days of electrical stimulus and increased by 50% after 4 days (Milkiewicz et al., 2003). Exercise is also a known stimulus for angiogenesis and in the event of a bout of exercise and/or training (depending on type and intensity) (Clifford & Hellsten, 2004), capillary growth and density was seen to increase; with more prominent capillary growth and higher VEGF-A levels seen in glycolytic fast twitch muscles than slow twitch oxidative muscles (Birot et al., 2003; Egginton, 2008; Hudlicka et al., 1992).

Exercise is seen as a potent stimulus for angiogenesis because it can initiate the many stimuli (mechanical and metabolic in nature) highlighted above to promote angiogenesis (Egginton, 2008; Haas & Nwadozi, 2015). With repetitive bouts of exercise, these stimuli can induce angiogenesis within days (Egginton, 2008; Haas & Nwadozi, 2015). With just one bout of exercise, many changes in the ECs' physiological environment occur to induce the first step towards capillary growth which when is continuously repeated, capillary growth is seen. Angio-adaptation in the skeletal muscle is described as dynamic processes that consist of capillary formation, capillary stabilization, or capillary regression in response to acute/chronic physiological or pathological conditions (Lemieux & Birot, 2021). The concept put forth is that under physiological conditions, there is a balance of pro- and anti- angiogenic genes that help to regulate angiogenesis (Olfert & Birot, 2011).

As outlined in Haas and Nwadozi review, with repeated bouts of exercise at the same intensity at the same level for seven days to two weeks time frame, there is a shift towards a more pro- angiogenic

phenotype and increase in capillary formation and density (Haas & Nwadozi, 2015). Immediately after a single (1 hour) bout of exercise, VEGF-A mRNA levels were increased around 4-fold and stayed elevated up to 4 hours post exercise in rats (Breen et al., 1996). In hypoxic conditions, the control rats showed increased levels of VEGF-A mRNA which increased more in rats performing a bout of exercise in hypoxia (Breen et al., 1996). When looking at daily repetition of an hour of submaximal running exercise in rats for a duration of 5 days, the mRNA levels of VEGF-A increased the greatest after the first day, with days two-five still having more VEGF-A levels than the control but less than the first day (Gavin & Wagner, 2001). In adult rat EDL muscles that were stimulated for 8 hours a day at 10 Hz, the capillary to fiber ratio increased by 15% after four days and after seven days it increased by approximately 50% (Hansen-smith et al., 1996). In both rabbit and rat muscles that were stimulated again for 8 hours a day for seven to fourteen days at 10 Hz, had an increase in capillary diameter and in the rat muscles, increase in capillary to fiber ratio after fourteen days (Myrhage & Hudlicka, 1978). The total capillary surface area per 100g of stimulated muscle in rat muscles increased by 29% after seven days and 46% after fourteen days (Myrhage & Hudlicka, 1978). When hindlimb unloading in rats was done for nine days, the oxidative muscle soleus was seen to have more capillary regression than the glycolytic muscle plantaris, and a shift towards anti-angiogenic pathway was seen in the soleus (Roudier et al., 2010).

After four to eight weeks of repetition of the same bout of exercise during training at the same intensity, capillary stabilization and a plateau in the growth of capillaries is observed (Haas & Nwadozi, 2015). In 14 male subjects, four weeks of 1 hour cycling training 3 times per week with a gradual increase from 60% to 68% of the individuals' VO_2 max showed a 23% increase in capillary to fiber ratio and 12% increase in capillary density (Hoier et al., 2012). Muscle interstitial VEGF-A protein increased during an acute bout of exercise (performed at same intensity for an hour) before and after four weeks of training (Hoier et al., 2012). There was also a transient increase in both pro and anti angiogenic factors in response to acute exercise before training (Hoier et al., 2012). With eight weeks of 1 hour run of hypoxia training in rats followed by an acute bout of exercise two days later, showed increased muscle capillarity but decreased

in VEGF-A, Flt-1, and Flk-1 (KDR) mRNA expressions compared to room air trained rats with same training protocol (Olfert et al., 2001). With respect to endurance training, the vastus lateralis muscle biopsies from 10 participants showed a 27% increase in capillary to fiber ratio and 16% increase in capillary density post-eight weeks of ergometer training compared to pre- exercise (Baum et al., 2015). This shows that angio-adaptation in the context of exercise and training results in capillary growth and stabilization, with lack of exercise resulting in capillary regression in oxidative muscles all caused by the balance between pro and anti- angiogenic molecules.

1.3.2. Two key pro- and anti- angiogenic molecules: VEGF-A and TSP-1

Angio- adaptation in the muscle is regulated by pro and anti angiogenic molecules, with the most studied molecule in the skeletal muscle being VEGF-A and thrombospondin-1 (TSP-1) (Roudier et al., 2010). As outlined previously, VEGF-A is a pro-angiogenic molecule from the VEGF family which are known for their role in angiogenic processes that occur throughout life. The VEGF A, B, C, D, E, and PlGF (placental growth factor) are all cysteine knot glycoproteins that are part of the VEGF family, with VEGF-A being predominantly studied (Neives et al., 2009; Ferrara, 1999). The human VEGF-A gene has different isoforms (121, 165, 189, and 206), with 165 being the major isoform (Ferrara, 1999). VEGF-A has been proven crucial for angiogenesis. When skeletal muscle VEGF-A was deleted in mice, capillarity was reduced as well as having impaired exercise capacity (Olfert et al., 2009). With one bout of exercise, protein and mRNA levels of VEGF-A were seen to increase in skeletal muscle (Birot et al., 2003). Basal levels of VEGF-A protein expressions increase with seven days of training in skeletal muscles, and decrease after twenty-eight days of training (Olenich et al., 2013).

The thrombospondin family consists of 5 proteins that are divided into subgroup A (TSP-1 and 2) and B (TSP-3, 4, and 5) due to their different structures and TSP-1 and 2 being the most studied (Zhang et al., 2020). The difference between TSP-1 and 2 are their amino acid sequences, causing differences in binding ligands (Zhang et al., 2020). With respect to TSP-1, it is produced in many different cells such as smooth muscle cells and endothelial cells and is an anti-angiogenic protein that can inhibit cell migration

and promote apoptosis in ECs (as reviewed by Olfert & Birot 2011). The three main receptors of TSP-1 are CD36, b1-integrin and CD47 (Olfert & Birot, 2011). TSP-1 can inhibit angiogenesis through complexes formed by its receptors CD36 and b1- integrin on the VEGF-A receptor VEGFR2 (KDR) in the endothelial cell membrane, causing inhibition of the VEGFR2 activation via phosphorylation (as reviewed by Olfert & Birot 2011). Another way TSP-1 can inhibit angiogenesis is by interacting with VEGF-A and degrading it (Olfert & Birot, 2011).

TSP-1 null mice showed greater capillarization and exercise capacity in the skeletal muscle (Malek & Olfert, 2009). With a single bout of exercise, TSP-1 mRNA levels were seen to increase in rat skeletal muscle; with three - five days of acute exercise, there was a loss of response of TSP-1 mRNA, and with eight weeks of training TSP-1 mRNA levels were restored (Olfert et al., 2006). Therefore, TSP-1 is truly important inhibitor of angiogenesis to maintain the balance in angio-adaptation. The expression of these angiogenic genes is under the control of transcriptional factors.

1.4. Transcriptional regulation of skeletal muscle angiogenesis

The process of angiogenesis is complex and is tightly regulated by multiple molecules. Transcription factors and coactivators involved in skeletal muscle angiogenesis include hypoxia inducible factor (HIFs), forkhead box O (FOXO), peroxisome proliferator-activated receptor-γ Coactivator-1α (PPGC-1α), and murine double minute 2 (MDM2).

1.4.1. FOXO1 and skeletal muscle angiogenesis

FOXOs are a family of transcription factors consisting of FOXO1, 3, 4, and 6; that can bind to different targets and play a role in regulating different pathways such as cell differentiation, apoptosis, organ development, and angiogenesis (as reviewed by Accili & Arden, 2004; Farhan et al., 2020). FOXO1 deleted mice embryos had smaller embryo size compared to the control and showed lack of blood vessels

(Hosaka et al., 2004). There were also vascular defects noticed in the heart of FOXO1deleted embryos, such as insufficient branching and lack of proper structure to coronary arteries (Hosaka et al., 2004). With regards to FOXO3 deletion, female mice showed lack of ovarian development and fertility issues while FOXO4 deletion did not show any atypical points (Hosaka et al., 2004).

Within ECs, FOXO1 and FOXO3 were observed to be most abundant; and ECs with overexpression of FOXO1 and FOXO3 showed significant impaired tube forming ability (Potente et al., 2005). FOXO1 was observed to play a role in promoting quiescence of ECs, FOXO1 activation in ECs increased the levels of S-2 hydroxyglutarate (S-2HG) which is a metabolic signalling molecule (Andrade et al., 2021). The increase of S-2 HG suppressed ECs proliferation and the ECs stopped in the G0/G1 phase of the cell cycle (resting phase of the cell) (Andrade et al., 2021).

With a single bout of exercise in mice, measured two hours post exercise bout, FOXO1 and FOXO3 levels of mRNA and protein increased (Slopack et al., 2014). After ten to fourteen days of repeated exercise, FOXO1/3 mRNA and protein levels decreased and TSP-1 (anti-angiogenic molecule) protein levels followed the same pattern as FOXO (Slopack et al., 2014). TSP-1 protein increased by 1.7-fold after an acute bout of exercise, and decreased after ten days of repeated exercise (Slopack et al., 2014). With EC deletion of FOXO, no increase in TSP-1 mRNA was seen after an acute bout of exercise, while a 20% increase in capillary to fiber ratio was observed after seven days of training (Slopack et al., 2014). In ischemic muscles, FOXO1 protein levels were increased at four- and seven-days (Milkiewicz et al., 2011). With nutrient stress and no flow conditions in cultured ECs, FOXO1 protein levels also increased (Milkiewicz et al., 2011). This suggests that in ischemia the lack of shear stress and reduced nutrient flow increases the FOXO1 protein levels (Milkiewicz et al., 2011). Peripheral arterial disease (PAD) is the reduction of blood flow and oxygen due to narrowing of peripheral arteries in the lower limbs, and is similar to an ischemic model. When comparing FOXO1 in PAD patients, FOXO1 protein levels was increased in PAD patients compared to healthy subjects; this was mirrored in ischemic mice where FOXO1 was increased as well (Roudier et al., 2013). TSP-1 was also increased in both PAD patients and ischemic mice

(Roudier et al., 2013). The deletion of FOXO in ECs of ischemic mice showed that the deletion of FOXO improved blood flow and induced angiogenesis (Roudier et al., 2013). In over- expression of FOXO1 in primary skeletal muscle microvascular ECs, TSP-1 mRNA increased and with chromatin immuno-precipitation of those cells showed that FOXO1 is a regulator of TSP-1 (Roudier et al., 2013). Hence, there is a lot of evidence to support that FOXO and FOXO1 play an important role in maintaining the balance of skeletal muscle angiogenesis through transcriptional regulation.

1.4.2. HIF-1α and skeletal muscle angiogenesis

Hypoxia inducible factor regulates cellular responses to hypoxia (Kumar & Choi, 2015). HIFs are heterodimers that are made up of one beta subunit (HIF -1β) and one of the three alpha subunits (HIF- 1 α, HIF- 2 α, HIF-3 α) and HIF-1 is the most studied within the HIFs family (Kumar & Choi, 2015). The HIF-1 α subunit is O_2 regulated; when low O_2 is sensed, HIF-1 α is stabilized and translocated to form a dimer with HIF -1β which is expressed constantly (Kumar & Choi, 2015; Lindholm & Rundqvist, 2016; Semenza, 2013). Strong basal level of HIF-1 α signal was observed in the skeletal muscle of mice that were in normoxia, which was increased in mice in hypoxia (Stroka et al., 2001). This suggests that HIF-1 α has a strong expression in normal air conditions and increases more in hypoxic conditions. The increases of HIF-1 α leads to the transcriptional regulation of certain genes like VEGF-A, GLUT 1, and GLUT 4 (Kumar & Choi, 2015). In terms of other factors, like shear stress and stretch, HIF-1 α (along with HIF- 2 α) was seen to be activated in response to stretch, not shear stress. In overloaded (to activate stretch) rat EDL muscles, HIF-1 α and HIF- 2 α both increased after four and seven days (Milkiewicz et al., 2007). In vitro, with skeletal muscle microvascular ECs exposed to 10% stretch, HIF-1 α and HIF- 2 α mRNA levels were increased (Milkiewicz et al., 2007). However, when these cells were exposed to 24 hours of elevated shear stress, HIF-1 α and HIF- 2 α mRNA decreased significantly (Milkiewicz et al., 2007).

Many studies have shown that in response to exercise, HIF-1 α is activated and aids in changes in gene expression (as reviewed by Lindholm & Rundqvist, 2016). After a single bout of knee extension exercise with reduced blood flow in healthy males, HIF-1 α protein levels increased and stayed up until six

hours post exercise, while HIF-1 α mRNA levels did not change (Ameln et al., 2005). VEGF-A mRNA increased 3 times more in the reduced blood flow subjects than the control subjects after an acute bout of exercise and peaked after two hours and stayed up until six hours as well (Ameln et al., 2005). In untrained HIF-1 α null mice, there was an increase in capillary to fiber ratio compared to untrained wild type mice (Mason et al., 2007). With endurance training, the wild type mice gained capillaries while the trained HIF-1 α null mice did not and already had an adaptation in capillary network that could not further be improved (Mason et al., 2007). In addition, HIF-1 α null mice had an upregulated GLUT4 expression at rest (Mason et al., 2007). This suggests that without HIF-1 α, there could be another mechanism involved that takes over and induces angiogenesis. In the skeletal muscle of elite endurance athletes, HIF-1 α expression is reduced with high levels of HIF-1 α inhibitors present (Lindholm et al., 2014). Therefore, HIF-1 α is seen to contribute to the transcriptional regulation of skeletal muscle angiogenesis through hypoxia conditions.

1.4.3. PGC-1α and skeletal muscle angiogenesis

PGC-1α is a transcriptional coactivator that interacts with transcription factors involved in different processes that include mitochondrial biogenesis, thermogenesis, fatty acid and glucose metabolism, and switching of fiber types in skeletal muscles (Liang & Ward, 2006; Mathai et al., 2008). As a transcriptional coactivator, the role of PGC-1α is to interact with transcription factors without binding to the DNA (Liang & Ward, 2006). PGC-1α coactivates the transcription factors known as the PPARs (PPAR- α, PPAR-δ, and PPAR-γ) (Liang & Ward, 2006). PGC-1α is expressed highly in the mitochondrial rich tissues and where oxidative metabolism is prominent such as the heart, skeletal muscle, and brown adipose tissue (Liang & Ward, 2006). In human vastus lateralis muscle, PGC-1α protein expression increased during prolonged exhaustive exercise, and remained upregulated for 24 hours after (Mathai et al., 2008). PGC-1α is also seen interacting with ERR α which increased VEGF-A expression in response to hypoxia independent of HIF pathway (Arany et al., 2008). The VEGF-A protein levels in the skeletal muscles of PGC-1α knockout mice was approximately 60-80% reduced compared to the wild type mice, with capillary to fiber ratio also being 20% lower than wild type mice (Leick et al., 2009). With five weeks of exercise training, skeletal muscle

VEGF-A protein levels only increased by 50% in wild type mice, while PGC-1α knockout mice showed no increases in VEGF-A protein levels with training (Leick et al., 2009). Therefore PGC-1 α is also an important coactivator in regulating angiogenesis alongside transcription factors FOXO and HIFs.

1.4.4. MDM2 and skeletal muscle angiogenesis

Murine double minute 2 (MDM2) is a E3 ubiquitin ligase first identified as an oncoprotein. As a P53 antagonist, MDM2 interacts with P53 tumor suppressor in a negative feedback loop (Moll & Petrenko, 2003). MDM2 has been seen ubiquitinating P53 to allow for its degradation to limit P53's suppressive functions (Moll & Petrenko, 2003). Independent of P53, MDM2 can mono and poly- ubiquitinate other transcription factors. Through these interactions, MDM2 can control gene expression in response to multiple stimuli in cancer and normal cells (Arena et al., 2018). In different cancer cells, co expression of MDM2 and FOXO resulted in reduction of FOXO4 expression (Brenkman et al., 2008). When MDM2 lacking its C- terminal RING finger domain (which characterizes it as an E3 ligase) was transfected in these cancer cells, it was unable to ubiquitinate FOXO4 (Brenkman et al., 2008). With co-expression and co-immuno-precipitation, FOXO4 and MDM2 was seen to directly bind to each other (Brenkman et al., 2008). In MCF 7 breast cancer cells, MDM2 was seen to be associated with FOXO3 through immuno-precipitation (Yang et al., 2008). As 293T cancer cells were transfected with different doses of MDM2, FOXO3 was reduced as the doses increased, and when the cells were treated with the proteasome inhibitor MG-132 (inhibits MDM2), FOXO3 levels were restored (Yang et al., 2008).

In MDM2 positive and P53 deleted MEF cells, VEGF-A protein expression and VEGF-A mRNA (under hypoxia) was higher than the double MDM2 and P53 knockout (Zhou et al., 2011). With regards to the tumour suppressor P53, the P53 knockout mice with induced hindlimb ischemia exhibited an increase in HIF-1 α and VEGF-A, along with increase in capillary density compared to the wild type (Pfaff et al., 2018). In cancer cells MDM2 expression was induced through hypoxia, increasing MDM2 expression but decreasing phosphorylation of MDM2 on serine 166 (Zhou et al., 2011). In high glucose conditions, MDM2

was observed to increase in ECs, and interact with DROSHA protein (plays a role in angiogenesis related microRNA maturation) and decreases DROSHA and VEGF-A protein expression, causing an anti-angiogenic shift (Lam et al., 2021). In skeletal muscle samples from humans, rats, and mice, after one bout of exercise, MDM2 did not increase however phosphorylated MDM2 on serine 166 (p-MDM2) and VEGF-A did (Aiken et al., 2016). Muscles and primary ECs that were stimulated with VEGF-A showed increases in p-MDM2 protein levels (Aiken et al., 2016). The overexpression of p-MDM2 in ECs showed 55% increase in MDM2 binding to FOXO1 and decreased FOXO1 dependent gene expression of TSP-1, shifting towards a more pro-angiogenic pathway (Aiken et al., 2016). In a nutrient stress or lack of shear stress environment, such as ischemia, p-MDM2 was reduced and affected the MDM2 and FOXO1 interaction. In microvascular ECs under nutrient stress, p-MDM2 was reduced by approximately 50%, and with day four of ligation in muscle in mice, p-MDM2 was reduced by 65% (Milkiewicz et al., 2011). With serum stimulation of these cells, p-MDM2 increased and FOXO1 decreased (Milkiewicz et al., 2011). When MG-132 (proteasome inhibitor) was treated on this serum stimulated cells, FOXO1 protein levels increased (Milkiewicz et al., 2011). Immuno-precipitation of MDM2 on ligated muscles showed reduced levels of FOXO1 pulled down compared to control (Milkiewicz et al., 2011). In cancer cells, MDM2 was seen to directly interact with HIF-1 α (Chen et al., 2003). Therefore, MDM2 can be seen to interact with transcription factors (like FOXO and HIF-1 α) that are known to play a role in angiogenesis (see Figure 1.6).

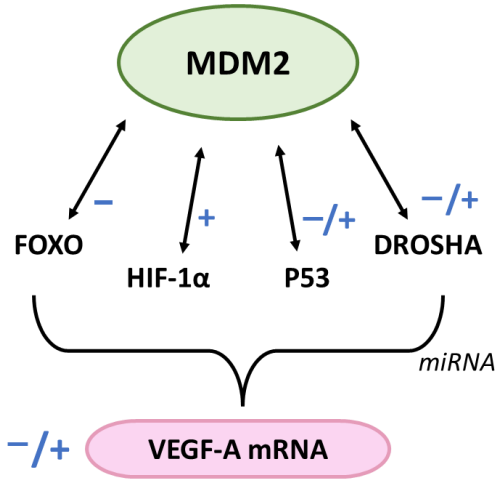


Figure 1.6: Schematic representation of the direct and indirect interactions of MDM2 in the context of angiogenesis. MDM2 can indirectly regulate VEGF-A expression through the direct interactions of transcription factors (HIF-1 α, FOXO), through the tumour suppressor P53, and through micro-RNA (miRNA) processing enzymes (DROSHA). MDM2 interacts with FOXO to decrease FOXO expression, which decreases an anti-angiogenic pathway. MDM2 interacting with HIF-1 α, increases its expression and shifts for a pro-angiogenic pathway. Depending on the cell type and condition, MDM2 interaction with P53 and DROSHA (through miRNAs) can either shift to a pro- or anti-angiogenic pathway. Image created on Biorender.

In the context of exercise, MDM2 increased with training and was observed to be crucial for muscle capillarization. In the vastus lateralis muscle of young and senior male subjects that performed six weeks of exercise training, platelet endothelial cell adhesion molecule-1 (PECAM- 1/ CD31) (marker for ECs capillarization) went up by 129% in the young subjects and 72% in senior subjects, alongside MDM2 which increased by 38% in young subjects and 81% in senior subjects (Roudier et al., 2013). Mice deficient for MDM2 reported 20% capillary regression compared to their wild type mice at basal level, independent of P53 (Roudier et al., 2012). With one bout of exercise, VEGF-A protein expression was increased by 64% in wild type mice compared to MDM2 deficient mice (Roudier et al., 2012). After eight weeks of exercise, the plantaris of wild type mice had an increased expression of MDM2 by 49% and capillarization by 24%, this was not seen in MDM2 deficient mice (Roudier et al., 2012).

The structure of MDM2 is the reason why MDM2 can play such complex roles in different pathways. The N terminal region of MDM2 binds to p53 and the C-terminal region contains the RING

(really interesting new gene) domain which promotes the E3 ligase activity (Fåhraeus & Olivares-Illana, 2014). Indeed, MDM2 has the potential to interact with many proteins, including proteins important for epigenetic processes such as histones (Fåhraeus & Olivares-Illana, 2014; Minsky & Oren, 2004). This allows MDM2 to also be an important epigenetic regulator, which will be explained further in the upcoming sections. Most recent, MDM2's capacity to interact with histones suggested a putative role of this E3 ubiquitin ligase as a modulator of gene expression through epigenetic processes (Fåhraeus & Olivares-Illana, 2014).

1.5. Epigenetics and exercise

Beyond the regulation of angiogenic genes through changes in the activity of transcription factors, there are additional mechanisms involved such as epigenetic regulations. To date most of the studies related to exercise and epigenetic processes were investigating transient gene activations and DNA methylation. As described above, exercise is one of the stimuli that can lead to skeletal muscle adaptations. These adaptations occur first as transient changes in gene expressions (McGee & Walder, 2017). Some gene expressions are upregulated/downregulated during exercise or post exercise which leads to changes in the muscle phenotype (McGee & Walder, 2017). These transient changes in gene expression could be due to epigenetic modifications. Epigenetics is a mechanism that regulates the expression of genes. Epigenetic processes refer to the changes in gene expression without changing the DNA nucleotide sequence (Widmann et al., 2019). Instead, they regulate gene expression through modifications of the chromatin structure. While miRNAs are considered as part of the epigenetic processes that can impact gene expression, epigenetic modifications that directly modulate the chromatin structure include histone modifications or DNA methylation (McGee & Hargreaves, 2019). DNA methylation occurs in the promoter regions on cytosines followed by guanosines (CpG) of genes, which leads to the condensing of the chromatin structure causing gene silencing to occur (Caiafa & Zampieri, 2005; Kanzleiter et al., 2015). Histone modifications

include post-translational modifications such as methylation, acetylation, and ubiquitination, on the lysine of histone tails, mostly seen on H3 and H4 (Rothbart & Strahl, 2014). Different modifications on different areas of the histone tail can result in either gene silencing or gene activation (Rothbart & Strahl, 2014).

Epigenetics in the context of skeletal muscles is still being explored (McGee & Hargreaves, 2019). Most studies have investigated the relationship between DNA methylation, histone modifications, and gene expression aiming to better understand how skeletal muscle adapts to exercise or exercise training. Studies are still aiming to determine whether epigenetic processes are crucial for skeletal muscle adaptation to exercise and training.

1.5.1. DNA methylation and exercise

DNA methylation causes gene silencing, specifically through the addition of a methyl group to the fifth carbon cytosine by enzymes called DNA methyltransferases (DNMTs) (McGee & Walder, 2017). As explained above, DNA methylation occurs usually on CpG islands which are areas enriched with promoters and first exons of genes, but has been seen occurring on non CpG areas as well (McGee & Walder, 2017). CpG islands are more frequently found in transcription start sites (TSS) and could be marked by histone modifications (Deaton & Bird, 2011). The histone mark H3K4me3, was seen to interact on CpG islands promoters which allows for activation. Conversely, the polycomb group proteins can silence the promoters on the CpG islands, through the polycomb repressive complex 2 (PRC2) complex mediated histone tri-methylated mark H3K27me3 (as reviewed by Deaton & Bird, 2011). These histone marks on the CpG island promoters can aid in the chromatin state to be either transcriptionally repressed or active (as reviewed by Deaton & Bird, 2011). Therefore, histone marks on the chromatin can affect gene expression by interacting on the TSS of CpG islands causing either the activation or the repression of genes.

Amongst DNMTs, DNMT1 is known for the maintenance of methylation patterns during DNA replication and mitosis (McGee & Walder, 2017). DNMT3 a/b are known for creating new methylation

patterns (McGee & Walder, 2017). DNA methylation is seen to interact with chromatin marks as well. The relation between DNA methylation patterns and the PRC2 complex H3K27me3 mark is mixed, they either work as antagonists or in synergy (as reviewed by Jeltsch et al., 2018). In cancer cells, genes with CpG islands enriched with H3K27me3 marks showed that they were lacking of DNA methylation, while H3K27me3 was depleted on DNA hypermethylated CpG islands (Brinkman et al., 2012). However, H3K27me3 marks were reduced in hypomethylated CpG islands in DNMT knockout mouse embryonic stem cells, suggesting that DNMTs and the PRC2 complex interacts (Brinkman et al., 2012). Therefore, genes that are fully silenced by DNA methylation result in less H3K27me3 marks, and on genes with DNA hypomethylation, H3K27me3 marks could be a way to transcriptionally repress genes as a compensatory repression (Brinkman et al., 2012).

Changes or dysfunctions in DNMTs leads to unusual DNA methylations and can link to physiological or pathophysiological changes (McGee & Walder, 2017). When studying DNA methylation patterns of PGC-1α (regulator of mitochondrial biogenesis) in type 2 diabetic males, PGC-1α in the vastus lateralis was hyper-methylated in non- CpG areas compared to healthy males and PGC-1α mRNA had a decrease of 38% (Barres et al., 2009). DNMT3b was increased in type 2 diabetic males compared to healthy males (Barres et al., 2009). This shows that through changes in DNMTs, DNA methylation can play a role in pathophysiological situations, in this case type 2 diabetes. DNA methylation patterns were also studied in the context of aging. In aged human skeletal muscle, there were more hyper-methylated genes than young skeletal muscles (Turner et al., 2020). In DNMT3a muscle specific deletion in mice, DNA methylation was seen to decrease after an acute bout of exercise, but with no changes in exercise or metabolic capacity (Small et al., 2021).

DNA methylation patterns can change due to environmental or physiological factors such as diet and exercise. With regards to exercise, studies have shown that DNA methylation is reduced on certain genes that help in skeletal muscle adaptations. With an acute bout of exercise, in the vastus lateralis muscle in males and females, DNA hypo-methylation was seen in metabolic genes such as PGC-1α (Barres et al.,

2012). The decrease in methylation led to the increase in those metabolic genes' mRNA (Barres et al., 2012). With ex vivo contraction of the soleus of mice, metabolic genes like PGC-1α increased three hours after contraction; forty-five minutes after contraction, the methylation on the promoters of PGC-1α were decreased (Barres et al., 2012). Skeletal muscle nuclei were seen to have epigenetic memory from prior training which increased its muscle adaptability through DNA methylation (Wen et al., 2021). Adult mice were put through eight weeks of progressive weighted wheel running training followed by detraining for twelve weeks (Wen et al., 2021). In the plantaris myonuclei and interstitial cells' nuclei, trained mice had acquired patterns of DNA hypomethylation and was retained in genes involved with muscle remodelling (Wen et al., 2021). Comparing newly trained mice to the four weeks retrained mice, prior trained plantaris muscles showed more hypertrophy than newly trained muscles, suggesting that there is a faster remodelling occurring in detrained muscles when resuming training (Wen et al., 2021). A study observing the differences in methylation between change of direction and straight-line high intensity running exercise protocols in active males, showed that change of direction exercise had a higher hypomethylation pattern across the genome, especially after thirty minutes of exercise (Maasar et al., 2021). VEGF-A was also hypomethylated within its promoter region after exercise for both running protocols (Maasar et al., 2021).

With regards to endurance training, quadricep muscle samples from mice were used to analyse transcriptional changes in response to four weeks of treadmill running (Kanzleiter et al., 2015). Negative correlation was observed between DNA methylation patterns and genes involved in metabolic pathways such as insulin signalling, oxidative phosphorylation, and pathways involved with growth and differentiation in endurance trained mice, indicating skeletal muscle adaptations to training (Kanzleiter et al., 2015). About 3020 genes were differentially expressed post training compared to pre training, with DNA methylation occurring on only 361 of those genes (Kanzleiter et al., 2015). This indicates that DNA methylation is not the only epigenetic process in charge of regulating genes in response to training. Different muscles also were noted to display different histone modifications at basal level and in response to a bout of exercise. For example, H3 acetylation was seen to be more abundant in the rat plantaris than in the rat

soleus in the promoter region of the PGC-1α gene (Kawano et al., 2015; Masuzawa et al., 2018). Altogether this leads us to consider that histone modifications should also be further investigated in depth in regards to gene regulation in exercise training.

1.5.2. Types of histone modifications

The nucleosome (see Figure 1.7) is constructed of DNA which is wrapped around two sets of histone proteins 2A, 2B, 3, and 4 (to make an octamer) (Even-Faitelson et al., 2016). Bunches of nucleosomes together form the chromatin structure, which is packaged into the nucleus (Even-Faitelson et al., 2016). When the chromatin is tightly wounded, causing strong electrostatic connection between negatively charged DNA backbone with the positive charged histones, the promoters of genes are inaccessible and is in a transcriptionally repressed state (Even-Faitelson et al., 2016). When the chromatin is loose, the promoters of genes are accessible to RNA polymerase II to allow for gene activation (Even-Faitelson et al., 2016). Post translational modifications (as listed above) on the chromatin can help activate or repress transcription of certain genes. Acetylation of histones occurs through an acetyl group being added to the histone tail, neutralizing the positive histone charge which decreases the electrostatic interaction between the DNA and histone, opening or loosening the chromatin (Peixoto et al., 2020). Histone acetylation is achieved by the balance of enzymes: histone acetyltransferases (HATs), which add acetyl groups, and histone deacetylases (HDACs), which remove acetyl groups (McGee & Walder, 2017). Histone methylation involves the addition of methyl groups (could be mono-, di-, tri-methylation) to different lysines or arginines of histones (Peixoto et al., 2020). Depending on the lysine/arginine, the methyl group and the number of methyl groups added could cause activation or repression of genes (Peixoto et al., 2020). Histone methylation occurs due to enzymes known as histone methyltransferases (HMTs) and the removal of methyl groups are done by enzymes known as histone demethylases (HDMs) (McGee & Walder, 2017). HMTs methylate lysines on the N terminal end of proteins contain a SET domain that has the enzymatic activity to catalyze methylation of lysine residues. Through lysine methylation, HMTs support chromatin condensation and gene silencing

(Bannister and Kouzarides, 2011; Cao et al 2022). The SET domains are named after the three genes it expresses, suppressor of variegation 3-9 (Su(var)3-9), polycomb group chromatin regulator enhancer of zeste (E(z)), and trithorax group chromatin regulator trithorax (Trx). (Cao et al., 2022; Dillon et al., 2005). Details on the PRC2 complex with focus on EZH2 will be discussed further for this thesis.

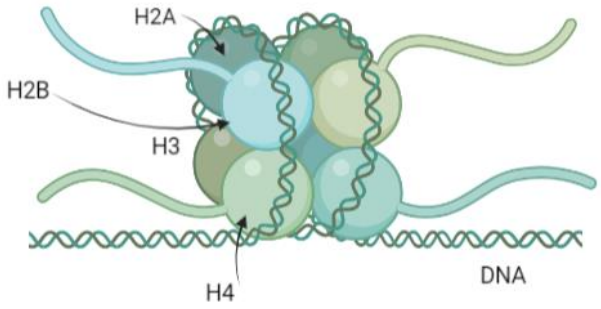


Figure 1.7: Diagram representing a nucleosome. Histone proteins come in two sets of H2A, H2B, H3, and H4 to make an octamer. The DNA wraps around these histone proteins to make a nucleosome, and multiple sets of nucleosomes together make the chromatin structure. Image created on Biorender.

Another post translational modification that occurs on histones are ubiquitination. Ubiquitination consists of proteins being covalently ligated to a ubiquitin molecule and targeted for degradation (Hershko & Ciechanover, 1998). This process is mediated by three enzymes E1, E2, and E3 ligases (Hershko & Ciechanover, 1998). E3 ligases like MDM2, have ubiquitin's C terminal linked to an amino group of the protein's lysine residue, and transfers ubiquitin on the substrate (Hershko & Ciechanover, 1998; Synder & Silva, 2021). E3 ligases bind specific proteins to ubiquitin (Hershko & Ciechanover, 1998). Ubiquitination can play a role in transcription, chromatin structure maintenance, DNA repair, and cancer (Cao & Yan, 2012). Ubiquitination can occur through either the addition of one ubiquitin molecule or many (poly-ubiquitin), with the most ubiquitinated histones being H2A and H2B (Cao & Yan, 2012). Histone 3 ubiquitination was seen to occur through the role of neural precursor cell expressed developmentally down regulated (NEDD4) in cancer cells in the presence of glucose (Zhang et al., 2017). Interestingly, NEDD4 overexpression increased H3 ubiquitination, that shifted the apparent molecular weight of H3 from the 17 kDA to 25 kDA, and the global abundance of two epigenetic modifications on H3, acetylation on lysine

residues 9 and 14 (H3K9 Ac and H3K14 Ac) (Zhang et al., 2017). Enzymes that remove ubiquitin molecules are known as deubiquitinating enzymes (DUBs). Environmental factors control the activity of these DUBs and control their capacity to catalyze the removal of ubiquitin molecules (Synder & Silva, 2021). DUBs are known to not only remove ubiquitin molecules, but to also recycle the ubiquitin molecule once its cleaved (Synder & Silva, 2021).

Histone modification	Function
H2AK4ac, H2AK5ac, H2AK7ac	Gene activation
H2AK119ub	Gene repression
H2BK5ac, H2BK11ac, H2BK12ac, H2BK15ac,	Gene activation
H3K4me1, H3K9me1, H3K27me1, H3K4me3	Gene activation
H3K9ac, H3K14ac, H3K27ac	Gene activation
H3K9me2, H3K9me3, H3K27me2, H3K27me3	Gene repression

Figure 1.8: Chart summarizing the basic histone code. Histone modifications are listed on the left and their observed function on the right, information retrieved from cell signalling histone modification table.

1.5.3. Current knowledge regarding exercise, skeletal muscle, and histone modifications

Although the effects of histone modifications from exercise is still a new area of research, studies have been able to show the complex interplay that occurs between histone modifications and resistance or endurance exercise in the skeletal muscle of rats, mice, and humans.

Since the chromatin structure is dynamic, there are variant histones that aid in controlling chromatin states (Huang & Zhu, 2014). H3.3 is a variant for H3 (different by four to five amino acids) and has the highest turnover (exchange of histones to new ones) at the promoter region (Huang & Zhu, 2014). H3.3 is associated with active histone modifications and is antagonistic to H3K27me3 which is a gene repressive histone mark (Huang & Zhu, 2014). With an acute bout of resistance exercise, the H3.3 variant (relative to total H3) was seen to decreased by 39% (Lim et al., 2020). When studying the effects of the duration and

amount of endurance exercise in the rat plantaris, H3.3 variant was seen to be downregulated with higher intensity running exercises which was followed by an increase in mitochondrial metabolism (Ohsawa et al., 2018). With the notion of epi-memory, eight weeks of endurance training followed by unloading on rats showed that in the plantaris, H3.3 variant was increased in rats with previous training compared to the control (Nakamura et al., 2017). The plantaris muscle also did not have atrophy after unloading and genes related to atrophy were reported to be less responsive to unloading in rats with previous training as well (Nakamura et al., 2017). Four weeks of endurance training increased the histone turnover in skeletal muscles. The H2B turnover was higher in the nucleosomes of genes that were upregulated by training in the myofibers. The overall abundance of H3.3 was increased with training, with less abundance in the nucleosomes of genes that were both down and up regulated by training (Ohsawa & Kawano, 2021). When measuring the gene silencing histone mark H3K27me3 after four weeks of endurance training, H3K27me3 increased in the myofibers (Ohsawa & Kawano, 2021).

When studying the difference between fast twitch and slow twitch muscles in histone modifications associated with gene activation, Kawano group observed that rat plantaris muscles showed higher H3K4me3 and H3 acetylation in all fast twitch genes than the soleus (Kawano et al., 2015). With the effects of overload in the rat plantaris muscles, expression of slow twitch genes were 1.7-2.3 folds higher, while fast twitch genes in the plantaris with overload did not change (Kawano et al., 2015). H3 acetylation was also seen to increase in slow twitch genes when the rat soleus muscles were overloaded (Kawano et al., 2015).

In human vastus lateralis muscles, genes important for muscle development, metabolism, and transcription factors were upregulated by 291% after an acute bout of resistance exercise. Yet, after ten weeks of resistance training, these genes were upregulated by 9.6% when compared to pre-training (Lim et al., 2020). The authors analyzed the distribution of acetylated H3 and H3K27me3 on the TSS of 16 genes that were upregulated by the acute resistance exercise. The acute bout of resistance exercise increased H3 acetylation by 235% and, H3K27me3 by 849% on those 16 genes, suggesting that histone modifications in

charge of activation and silencing are both being upregulated to possibly maintain a balance (Lim et al., 2020). After ten weeks of resistance training, the distribution of H3K27me3 did not change; however, an enrichment in H3 acetylation was observed on the TSS of those upregulated genes. Unfortunately, the authors did not report whether acute resistance exercise or resistance training changes the distribution of H3 modification on downregulated genes.

With six weeks of endurance training on the ergocycle for 60 minutes for five days a week at 70% max aerobic capacity, vastus lateralis muscles from eight previously sedentary males showed the upregulation of 641 genes and downregulation of 176 genes in the post- trained muscle compared to pre-trained muscle (Williams et al., 2021). The post-trained muscles had an enrichment in histone modifications H3K4me1 and H3K27ac in the enhancer region of genes that were upregulated by the training program (Williams et al., 2021). With an acute bout of endurance exercise in the tibialis anterior muscle of mice, the transcription of 422 genes was promoted by exercise. These genes harboured an increase in both H3K27me3 and H3K4me3 marks on their TSS (Shimizu & Kawano, 2022).

With regards to enzymes that methylate or acetylate lysine residues on histones, HDACs are the most commonly studied with exercise. With 60 minutes of cycling, the human vastus lateralis muscle showed that HDAC 4 and 5 were exported from the nucleus, which removes their ability to cause transcriptional repression by their inability to remove acetyl groups (McGee et al., 2009). Since the study of epigenetics is still an emerging concept in the context of exercise, there are many gaps of knowledge. There are many studies that look into histone acetylation with exercise; however, histone methylation and gene silencing in terms of exercise and angiogenesis in the skeletal muscle is still yet to be discovered.

1.6. The role of EZH2 in the skeletal muscle and angiogenesis

The polycomb group proteins (PcG) are known as key regulators of cells in early development. These proteins are observed to play a critical role in cellular processes, cell cycle control, and cancer (Morey

& Helin, 2010). These proteins are also required for proper expression of homeotic (HOX) genes during the developmental stages (Morey & Helin, 2010). The PRC1 and PRC2 complexes are the two main complexes of PcG that have a catalytic function that leads to transcriptional repression (Morey & Helin, 2010). The PRC2 complex is made up of suppressor of zeste 12 (SUZ12), embryonic ectoderm development (EED), and enhancer of zeste homolog 2 (EZH2) (Morey & Helin, 2010). EZH2 is the catalytic subunit of the PRC2 complex, and as an HMT, it places di- or tri-methylation marks on histone 3 lysine 27 (H3K27me2/3) which is a repressive histone mark (Morey & Helin, 2010). HDAC1 and 2 were also seen to be part of the PRC2 complex; however, they are not needed for the HMT activity (Morey & Helin, 2010). Histone 2A on lysine 119 mono-ubiquitination (H2AK119ub) is catalyzed through the PRC1 complex, which is also a repressive histone mark (Morey & Helin, 2010).

EZH2 is observed to be critical for early development as deletion from mice embryos was shown to be lethal (O'Carroll et al., 2001). Mice embryos that had EZH2 deletion terminated development after implantation or could not complete their gastrulation stage (O'Carroll et al., 2001). In wild type mice embryos, EZH2 is noticed to be up regulated upon fertilization (O'Carroll et al., 2001). With regards to cancer and tumour angiogenesis, EZH2 is studied extensively. The dysregulation of EZH2 was observed to accelerate cell proliferation and prolong cell survival which led to the increase of cancer development (as reviewed by Duan et al., 2020).

With regards to skeletal muscle, angiogenesis, and exercise, EZH2 is still yet to be researched. Recent studies showed the relationship between skeletal muscle differentiation and EZH2. The expression of EZH2 was measured in mouse embryos from days 9.5-15.5 and showed that EZH2 was highly expressed in most areas of the embryos at day 9.5, while by day 11.5 EZH2 expression had significantly decreased (Caretti et al., 2004). In cultured mouse muscle satellite cells, EZH2 mRNA was highly expressed in proliferating satellite cells and decreased as differentiation continued (Caretti et al., 2004). In C2C12 myoblasts transfected with either EZH2 or EZH2 Set domain deficient (removes HMT activity), cells with EZH2 transfection resulted in impaired differentiation while cells with EZH2 Set domain deficient

transfection did not (Caretti et al., 2004). This suggests that the HMT activity of EZH2 is the reason for the inhibition in muscle differentiation. In human primary myoblasts, EZH2 was seen to be expressed in proliferating myoblasts and was downregulated after differentiation as well (Stojic et al., 2011). EZH2 was also noted to be required for adult satellite cell maintenance. Mice that had a deletion of EZH2 in their satellite cells had less of muscle postnatal pax7 cells (important for satellite cell specification and survival), reduced muscle mass, and had problems with proper muscle regeneration (Juan et al., 2011). The relationship between EZH2 and the epigenetic modification miRNA was studied and showed that mIR-214 (found repressed by PcG proteins in undifferentiated skeletal muscle cells) reduced the EZH2 protein levels and increased muscle differentiation and gene expression in C2C12 cells (Juan et al., 2009). These studies highlight the critical role of EZH2 as an HMT in both cancer and early development of skeletal muscle cells. In terms of exercise and skeletal muscle angiogenesis, EZH2's epigenetic functions need to be investigated to further understand the full potential role EZH2 could play in gene expression and exercise in skeletal muscle.

1.7. The control of epigenetic processes by MDM2

The oncoprotein MDM2 is an E3 ubiquitin ligase, and can be seen to have epigenetic functions as it can ubiquitinate histones, interact with transcriptional repressors, and interact with transcription factors which will lead to changes in gene expressions. Majority of the epigenetic research on MDM2 is in the context of cancer. MDM2 can be controlled by post-translational modifications through controlling its conformation (Fåhraeus & Olivares-Illana, 2014). Ternary complexes can function to change the substrate specific of MDM2 (Fåhraeus & Olivares-Illana, 2014). For example, Notch 1 is seen to form a ternary complex with MDM2 which then promotes the ubiquitination and signalling of the Notch 1 pathway (as reviewed by Fåhraeus & Olivares-Illana, 2014). MDM2 can bind to the chromatin and to other epigenetic related proteins. In a study done on cancer cells, MDM2 was seen to bind to the chromatin with and without

P53 (Riscal et al., 2016). MDM2 can mono-ubiquitinate histone 2A and 2B in cancer cells as well; MDM2 can cause transcriptional repression due to its RING domain, therefore, the mono-ubiquitination of H2A and H2B by MDM2 can cause gene silencing (Minsky & Oren, 2004). In cancer cells, MDM2 was associated with androgen receptor and HDAC1 to mediate the ubiquitination of these proteins (Gaughan, 2005). Cancer cells under oxidative stress showed that the protein kinase human MPS1 phosphorylated MDM2 which then promoted the ubiquitination of H2B and led to chromatin loosening and gene activation (Yu et al., 2016). In vascular smooth muscle cells, MDM2 was shown to ubiquitinate HDAC1 which led to HDAC1 degradation and facilitated vascular calcification (Kwon et al., 2016). The deubiquitinase enzyme USP49 (known to deubiquitinate H2A) was observed to suppress MDM2 activity in cancer cells (Shi et al., 2022). The overexpression of USP49 suppressed MDM2 by directly binding to the promoter of the MDM2 gene and increased the P53 tumour suppressor's target genes (Shi et al., 2022). In mouse embryonic fibroblasts (MEFs) and cancer cells, Tip60 which is a HAT and a cofactor of transcription, was shown to interact with MDM2 through co-immunoprecipitation and the overexpression of Tip60 lead to an increase in MDM2 protein levels (Dohmesen et al., 2008). The E3 ligase NEDD4 can interact physically with MDM2 on the RING domain to stabilize it (Xu et al., 2015). In MEFs, MDM2 ubiquitination increased as NEDD4 overexpression increased (dependent on time and concentration) (Xu et al., 2015). The co-immunoprecipitation of endogenous proteins showed that MDM2 directly interacts with the histone methyltransferase protein EZH2 from the transcriptional repressors PRC2 complex (Wienken et al., 2016). The knockout of MDM2 and EZH2 in MEFs suggested that MDM2 and EZH2 can co regulated certain genes. In MEFs, MDM2 knockout had the same effect on gene expression that knocking out EZH2 had (Wienken et al., 2016; Wienken et al., 2017). Results from a targeted chromatin immunoprecipitation (ChIP) revealed reduced H3K27me3 and H2AK119ub levels on the homeotic transcription factors (HOX) genes in the MDM2 MEFs knockout (Wienken et al., 2017). In their supplemental data, the authors show that some angiogenesis related genes were affected by MDM2/ EZH2. MEFs with MDM2 knockout showed changes in KDR (VEGFR2, upregulation) and Notch 1 (downregulation) gene expression (Wienken et al., 2016), both known to play a role in skeletal muscle angiogenesis (see paragraph 1.3 above). MDM2 as an

E3 ligase has many functions that range from angiogenesis to epigenetics and is seen playing a role in both physiological and pathophysiological conditions.

Since MDM2 is a complex protein with different roles, this thesis aims to investigate the impact of endurance exercise and training on histone modifications putative regulated by the MDM2 and EZH2 pathway and to delineate whether it can impact skeletal muscle angiogenesis.

Chapter 2: Gaps of knowledge, scientific question, and research objectives

2.1. Gaps of Knowledge

One single bout of exercise can increase both pro- and anti- angiogenic signals in the skeletal muscle. Repeated bouts of exercise at the same intensity over two weeks (short-term training), will enable a pro-angiogenic shift to allow for exercise- induced skeletal muscle angiogenesis to occur (Haas & Nwadozi, 2015). After about four to eight weeks of the same intensity training, capillary growth stabilizes due to capillarization matching the blood supply to the metabolic needs of the contracting myofibers (Haas & Nwadozi, 2015). This silences the pro-angiogenic signals and stops capillary growth and corresponds to the prolonged training angiogenic plateau. The role of epigenetic mechanisms in the context of exercise-induced skeletal muscle angio-adaptation is unknown. As discussed above, histone mark H3K27me3 can silence gene expression. To our current knowledge, there is no clear understanding of how H3K27me3 is affected during the time course of exercise training in the skeletal muscle and if this could support the prolonged training angiogenic plateau.

MDM2 has many functions that depend on its interaction with certain proteins (Fåhraeus & Olivares-Illana, 2014). Our laboratory has shown that MDM2 was a key protein in exercise- induced skeletal muscle angiogenesis (Roudier et al., 2012) and can regulate the expression of transcription factors and angio-adaptive molecules such as VEGF-A and TSP-1. Our lab has also observed that MDM2 increases with six weeks of endurance training (Roudier et al.,2013). Recent studies have suggested that MDM2 has epigenetic functions, including the regulation of H3K27me3 through direct interaction with EZH2 (subunit of PRC2 complex) (Potente et al., 2005; Wienken et al., 2016). MDM2 deletion in MEFs exhibited an upregulation of KDR, which was mirrored in EZH2 deleted MEFs (Wienken et al., 2016); which could impact the angiogenesis process. Yet, the contribution of Mdm2 and EZH2 to H3K27me3 silencing marks, and particularly on KDR, has never been investigated in the context of skeletal muscle angiogenesis.

2.2. Scientific question and research hypothesis

Could Mdm2 and EZH2 regulate physiological muscle angiogenesis in skeletal muscle via the establishment of the silencing epigenetic mark H3K27me3?

My hypothesis is that endurance exercise or training modifies the abundance of post-translational modifications of histone H3, including H3K27me3, regulated by the MDM2 and EZH2 pathway to promote these marks on angiogenic genes including KDR (Figure 2.1). This scientific question and hypothesis will be addressed through the following research objectives.

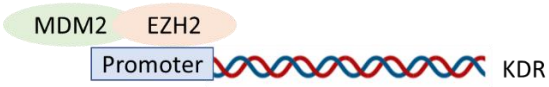


Figure 2.1: Diagram of MDM2 and EZH2 pathway acting on the promoter region of the KDR gene. MDM2 and EZH2 epigenetic marking causing H3K27me3 silencing marks to act on the promoter of KDR to regulate the angiogenic gene in the context of exercise.

2.3. Research objectives

Objective 1: To characterize whether short-term training (fourteen-day) alters the response of histone modifications and histone modifiers to an acute bout of exercise.

To test the impact of short-term training on the response to acute exercise, C57BL6 female mice were endurance trained for 14 days or remain sedentary before being subjected to an acute bout of exercise. Muscle collection was performed before, immediately after acute exercise, or 3 hours post recovery from acute exercise, creating 6 groups of mice, as indicated below:

Sedentary control	Sedentary acute	Sedentary 3-hour recovery
Trained control	Trained acute	Trained 3-hour recovery

The gastrocnemius muscle was used to analyse the levels of histone modifications and histone modifiers.

Objective 2: To characterize the effect of long- term endurance (nine weeks) training on the histone modifications potentially regulated by histone modifiers MDM2 and EZH2.

To test the impact of long- term training C57BL6 female mice were endurance trained for 1 hour 5 days a week for 9 weeks or remain sedentary. Muscle collection was performed 72 hours after last bout of exercise. Protein and mRNA expressions will be measured predominantly in the gastrocnemius muscle, and in the plantaris and the soleus muscle.

For objectives 1 and 2, the global abundance of the silencing mark H3K27me3 in response to acute exercise, short-term and long-term endurance training will be measured by western blots, to assess whether an increase of MDM2 positively correlates with changes in H3K27me3. The presence of H3K27me3 in a DNA loci influences the establishment of other epigenetic marks, such as H2AK119Ub and H3K4me3 (Wienken et al., 2016; Kanki et al., 2022). The impact of exercise training on these epigenetic marks will also be investigated. Histone modifiers EZH2 and NEDD4 interact with MDM2, their protein expressions will also be measured.

Objective 3: To determine the impact of endurance training on the expressions of angiogenic genes regulated by MDM2 and EZH2.

MDM2 could directly interact with EZH2 and both were observed to regulate the angiogenic gene KDR in MEFs (Wienken et al., 2016), *Kdr* mRNA levels in the gastrocnemius and plantaris of long-term endurance trained (nine weeks) C57BL6 female mice will be measured to determine potential correlation between MDM2, H3K27me3, and *Kdr* mRNA expression levels. EZH2 inhibitor GSK343 will be used to inhibit

EZH2 in mouse skeletal muscle microvascular endothelial cells in vitro (mSMECs) to determine whether EZH2 activity impacts KDR mRNA expression.

Objective 4: To specifically assess histone modifications involved in the regulation of the angiogenic gene KDR.

H3 is tri-methylated on lysine 27 (H3K27) through the action of EZH2 which induces gene silencing. Analysis of the amount of histone silencing mark H3K27me3, on the gene KDR, will be done in sedentary and long-term endurance (9 weeks) trained gastrocnemius muscle of C57BL6 female mice.

Chapter 3: Methods

Ethical approval:

Long term training and care of C57BL6 female mice were done by Dr. Abdul Sater's lab. Surgery and muscle collection for long term trained mice were done by our lab and Dr. Abdul Sater's lab. Acute exercise training and care of C57BL6 female mice were done by our lab and Dr. Olivier Birot's lab. Surgery and muscle collection for acute exercise trained mice were done by our lab and Dr. Birot's lab. COX-IV protein expression in the 9 weeks trained gastrocnemius muscle was measured by previous lab student Akash Mudur (Figure 4.5A). MDM2 and EZH2 protein expressions in the 9 weeks trained gastrocnemius was measured by previous graduate student Manpreet Gulri (Figure 4.5B). Dr. Emilie Roudier and Dr. Abdul Sater received ethical approval from York University Committee Animal Care.

3.1. Long term training

Fourteen 7 weeks old female C57Bl6 mice were divided in a trained vs. sedentary group both at n=7. Standard chow was used to feed all mice and sedentary mice were allowed to roam their cages. Mice were acclimatized to treadmill exercise for five days at 20m/min prior to the nine-week training program. For the nine-week training program, mice ran for one hour per day, for five days per week. As the training program continued, the speed gradually increased up to 30 m/min: week one – 20 meters/min, week two - 22m/min, week three - 24.2m/min, week four - 26.6m/min, weeks five-nine - 30m/min, representing speeds of 1.2 kmph to 1.8 kmph. The mice ran at approximately 70-75% of their VO2 max, which is categorized as moderate to vigorous intensity. After 9 weeks, sedentary and trained 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.

3.2. Acute exercise training

Forty 8 weeks old female C57Bl6 mice were divided into two groups: sedentary and trained. Each group was further subdivided into three exercise conditions, 1 hour acute (n=6), 1-hour acute exercise with 3-hour recovery (n=7), and no 1-hour acute exercise/ control (n=7). All mice were fed standard chow and were allowed to roam their cages. All mice were acclimatized one week (starting at 2 cm/s and went up to 10 m/min for 10 minutes/day) before the acute exercise protocol. For the training group, fourteen days of daily 1-hour acute exercise of treadmill running was performed, slowly increasing the speed from 10m/min to 24m/min then maintaining the speed at 5% incline. There was a 24-hour washout period from the last training period before the performing the acute bout of exercise. Food and water were removed one hour prior to the acute bout of exercise. All mice, except sedentary control/trained control, performed 1-hour acute exercise at max speed of 24 m/min at 5% incline. The mice were anaesthetized (isoflurane/oxygen inhalation) for immediate muscle collection after the 1-hour acute exercise. Several lower limb muscles (including the soleus, gastrocnemius, and plantaris) were removed, weighted, and snap-frozen in liquid nitrogen.

3.3. Cell culture and treatment

Primary mouse skeletal muscle microvascular endothelial cells (mSMECs) from C57Bl6 mice were purchased from Cedarlane Labs (C57-6220). Mouse SMECs were cultured at 37 °C and 5% CO_2 in complete mouse endothelial cell medium (Cell Biologics; cat# M1168), n=6 for protein and n=4 for RNA analyses. Cells from passage 7 were used and then plated on 60 mm dishes or 6 well plate coated with 1.5% gelatin at a density of 200,000 cells when at passage 9. Cells were then treated with EZH2 inhibitor GSK343 (Cell signalling; cat# 66244S) for 48 hours at a concentration of 1µM. Control cells were treated with DMSO (Sigma- Aldrich; cat# D2650). RNA and protein were collected after 48 hours from control and GSK343 treated mSMECs. RNA was collected in 700 µL of QIAzol lysis reagent (cat # 79306, Qiagen,

Toronto, Canada) and protein was collected in 150 µL of complete Radio Immuno-Precipitation Assay lysis (RIPA buffer) (50 mM Tris Base, 100mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 5mM EDTA, pH 8.0) with protease inhibitor 1mM PMSF, phosphatase inhibitors 1 mM NaF and Na_3VO_4, a protease inhibitor cocktail (Complete mini, Roche Diagnostics; cat #049006845001) and a phosphatase inhibitor cocktail (Complete mini, Roche Diagnostics; cat #11836153001). Protein and RNA isolation done as outlined below.

C2C12 differentiated myotubes protein extracts were received from Dr. Birot's Lab. C2C12 differentiated myotubes were cultured and extracted according to Lemieux et al., 2022.

3.4. Protein extraction

For skeletal muscle protein extracts; protein extracts were collected from plantaris and gastrocnemius muscles of 9 weeks of trained C57BL6 female mice. 30-50 mg muscle tissues were collected and submersed in complete RIPA buffer. Muscles were lysed twice using the tissue homogenizer Retsch MM400 (Mixer Mill, Retsch GmbH, Haan, Germany) at for 30 pulses/second for 30 seconds. Muscle homogenates were sheared in the Bioruptor for 8 cycles for 15 seconds ON and 45 seconds REST. The generated homogenate was centrifuge at 4°C for twenty minutes at 12,000 g, and the supernatants were collected to serve as homogenates. Skeletal muscle protein extracts were quantified using BCA methods as outlined below.

For mSMECs protein extracts, cells were washed with cold 1X PBS three times and then collected in 150 µL complete RIPA buffer. Cells were then sheared using the Bioruptor for 5 cycles with 30 seconds ON and 30 seconds REST. After shearing cells were centrifuged for fifteen minutes at speed 12,000g at 4°C.

3.5. Protein concentration determination

Protein concentrations were determined using the bicinchoninic acid assay (BCA). Muscle and cell protein extracts were loaded in triplicates and measured at 562 nm absorbance on Gen5 plate reader Biotek. Standard curve plotted by bovine serum albumin (A7906 Sigma-Aldrich). Cell protein samples were diluted 1:5 with ddH_2O and muscle protein samples were diluted 1:20 with ddH_2O. BCA reagents bicinchoninic acid (Sigma-Aldrich; cat# 32 B9643) and copper (II) sulfate solution (Sigma-Aldrich; cat# C2284) were used and samples with BCA reagents were incubated at 37 °C for thirty minutes.

3.6. RNA isolation

Muscle RNA isolation and purification was performed using RNeasy fibrous tissue mini kit (Qiagen, cat# 74704). 15 mg of gastrocnemius and plantaris muscles were lysed in RLT buffer from kit and homogenized in tissue homogenizer Retsch MM400. Cell mSMECs RNA isolation and purification was performed using the miRNeasy micro kit (Qiagen, cat #217004). RNA was quantified using Gen5 plate reader Biotek at 1:20 dilution 260/280 nm absorbance.

3.7. Western blotting

20 µg of protein samples were denatured and loaded on SDS- PAGE, blotted on 0.2 µm or 0.45 µm nitrocellulose membrane. Membranes were blocked with 5% fat-free milk. Antibodies used were; H3K27me3: predominantly Diagenode (C15410195, Burlington, Canada), Diagenode (C15410069, Burlington, Canada), and Cell signalling (C36B11, Whitby, Canada), PECAM (CD31): Invitrogen CD31 polyclonal antibody (PA5- 16301, Burlington, Canada), a/b Tubulin: Cell signalling (21485, Whitby, Canada), MDM2: rabbit (D1V2Z) Cell signalling (86934, Whitby, Canada) and 2A10 non- commercial mouse (kindly provided by Dr Mary Ellen Perry NIH), EZH2: (DC29) Cell signalling (5246, Whitby,

Canada), H3: mouse Cell signalling (3638, Whitby, Canada) and Ubiquitin: mouse (P4D1) Cell signalling (3936, Whitby, Canada), COXIV: Cell signalling (4844S, Whitby, Canada), NEDD4: Cell signalling (2740S, Whitby, Canada), H3K4me3: Cell signalling (9727S, Whitby, Canada), p-EZH2: Cell signalling (7888S, Whitby, Canada), H3K9/14ac: Diagenode (15410200, Burlington, Canada), H2AK119ub: Cell signalling (8240T, Whitby, Canada), H2A: Cell signalling (2578, Whitby, Canada).

3.8. Immuno-precipitation

30-50 mg of control gastrocnemius muscles' proteins were extracted and sheared as outlined above. Day one consisted of washing 20 µL protein magnetic A beads (cat# 73778, Cell signalling, Whitby, Canada) in 500 µL of RIPA without inhibitors, then preclearing 200 µL of lysate (600 µg of muscle protein and complete RIPA) with washed magnetic A beads for twenty minutes at room temperature. Lysate was then incubated overnight on rotation at 4 °C with 5 µg of either H3K27me3 (cat# C15410195, Diagenode, Burlington, Canada) or rabbit IgG (cat# C15410206, Diagenode, Burlington, Canada) antibodies. Day two consisted of washing 20 µL protein magnetic A beads with 500 µL of RIPA without inhibitors and then transferring the lysate with antibodies into pre-washed magnetic beads. Incubate the samples for twenty minutes on rotation at room temperature. The magnetic beads fixed with the immunocomplex was then separated using a magnetic rack. The immunocomplex was washed five times in 500 µL RIPA without inhibitors and kept on ice in between washes. 30 µL of 1/5 DNB: RIPA was used to resuspend the immunocomplex and then followed western blotting procedure with 20 µg of control muscle as input. Anti-ubiquitin mouse and anti- H3 mouse antibodies were used for immuno-blotting.

3.9. RT-qPCR

Reverse transcription was done using 500 ng of RNAs extracted from muscles or cells using the high-capacity RNA to cDNA kit (cat# 4387406, Thermofisher Scientific, Burlington, Canada). Real- time qPCR was run using Taqman probes (listed below) and Taqman fast advanced master mix (cat# 4444557, Thermofisher Scientific, Burlington, Canada) in triplicates. PCR was run for 40 cycles of denaturation at 95°C for 3 seconds, and annealing and extension at 60°C for 30 seconds. KDR, NOTCH, and HPRT were measured in RNA extracts from muscles and cells.

List of TaqMan probes used to measure RNA transcripts.

Assay ID	Target	Sequences
Mm01545399	HPRT	GGACTGATTATGGACAGGACTGAAA
Mm01222421	KDR	CATCCTAGAGCGCATGGCACCCATG
Mm00627185_m1	NOTCH-1	GCCGCAAGAGGCTTGAGATGCTCCC

3.10. Chromatin immuno- precipitation followed by qPCR

30-60 mg of nine weeks trained gastrocnemius muscles were homogenized in cooled PBS with protease inhibitor cocktail (cat # 04900684500, Complete mini, Roche Diagnostics) and a phosphatase inhibitor cocktail (cat # 11836153001, Complete mini, Roche Diagnostics), 5 mM of butyrate, and 10 mM of GSK-J4 (inhibitor of H3K27me3 eraser, cat#14071, Active Motif, Burlington, Canada). After centrifugation at 12,000 g, the pellets were fixed with 1% paraformaldehyde for fifteen minutes at room temperature to allow for cross link; to stop the cross link, 10X glycine was added and incubated for five minutes. The pellet was resuspended in ChIP lysis buffer from Thermofisher ChIP kit (cat# 26157, Thermofisher, Burlington, Canada) and was sheared in the Bioruptor for 10 cycles for 15 seconds ON and 45 seconds REST, to have DNA fragments between 450-900 base pairs (bp) as verified by DNA gel (see supplemental data; Figure 6.6), and PCR purification was later done on PCR products to measure the

fragment sizes (supplemental data; Figure 6.6). After centrifugation at 12,000 g for twenty minutes, chromatin was collected and diluted 1:5 (not input samples) in ChIP buffer solution from Thermofisher ChIP kit and was incubated with either anti-H3K27me3 antibody (cat# C15410195, Diagenode, Burlington, Canada) or anti-H3 pan antibody (cat# C15200011, Diagenode, Burlington, Canada) at 6 µg concentration overnight at 4°C on rotation. Samples had 20 µL of protein A/G ChIP grade magnetic beads from Thermofisher ChIP kit and incubated for two hours at 4°C on rotation. Samples were then washed three times with a low salt wash and one time with a high salt wash from ChIP kit. The samples with beads were resuspended in 150 µL of elution buffer from Thermofisher ChIP kit and was incubated at 65°C for forty minutes. After collecting the supernatant, RNAse A (ChIP kit) was added and incubated at room temperature for fifteen minutes, and then proteinase K (ChIP kit) was added and incubated at 65°C for two hours. DNA fragments were then purified using spin columns provided in the Thermofisher ChIP kit. Nuclease free water was used to eluate the DNA fragments; 50 µl of nuclease free water was added to the columns, eluates were collected after two minutes of centrifugation at 10,000 g.

2.5 µL of undiluted ChIP products or 2.5 µl of the 20% INPUT were used to quantify the presence of DNA fragments corresponding to the TSS region of the KDR promoter using semi-quantitative PCR (qPCR). Briefly, ChIP products or INPUT were mixed with Thermofisher master mix and custom designed probes for KDR gene. Two Custom Taqman probes were originally designed and purchased from Thermofisher. These probes were designed to ensure amplification of the TSS regions of the KDR gene where CpG islands were previously reported to be enriched in H3K27me3; according to Wienken et al., the TSS region was located on Chr5 starting at 76374433 and ending at 76373341. The efficiency of the qPCR was tested by ChIP products, generated using the anti-H3 antibody as a positive control. CT values obtained using the KDR ChIP 1 probe showed more relying data than KDR ChIP 2. Therefore, qPCR was run on all samples using KDR ChIP probe 1, for 40 cycles of denaturation at 95°C for 3 seconds, and annealing and extension at 60°C for 30 seconds.

Taqman probe used to measure KDR:

KDR-ChIP1	Sequence	Tm	Size of amplicon (bp)
Forward Primer	CATGACAAAACCCACCCAGAT	58	68
Reverse Primer	TGGACCTAAGGATAGGCATTCTGT	59	
Probe	CCATGTGGATAAATC	69	
KDR-ChIP2	Sequence	Tm	
Forward Primer	GGCCATGGACGTGGCTTA	59	70
Reverse Primer	GGAGATTGAGCTCAGGACATCAG	59	
Probe	AAGTAAAGTGCTTCCTGCTGA	69	

3.11. PCR purification and DNA gel

qPCR reactions were first run using PCR probes and diluted INPUT products to ensure that the probes generated the correct size of amplicons (as listed above). The purification of the products of PCR were performed using Pure link PCR purification kit (cat# K310001, Thermofisher, Burlington, Canada). PCR purified products were loaded onto a 2% agarose gel using ethidium bromide and 1X TE buffer (100 mM Tris base, 10mM EDTA, pH 8), confirming the correct size of amplicon. DNA gel used 100 bps DNA ladder (cat# N3231S, New England Biolabs, Whitby, Ontario) and DNA loading gel (cat# B7025S, New England Biolabs, Whitby, Ontario).

3.12. Determination of % of INPUT

The enrichment in H3K27me3 marks was estimated using the percentage of input (% INPUT) as described by Solomon et al., 2021. The % INPUT can be calculated once quantification cycles (Cq), also called cycle threshold (CT), of the INPUT (Cq^{IN}) and the ChIP product (Cq^{CHIP}) are estimated; however, but it needs to take into consideration the dilution factor (DF). ChIP protocol was performed at constant volume. Taking in consideration the initial volume of lysate used for the IP and for the input and the dilution

performed to have an INPUT at 20%, the INPUT was 20 times more diluted than the ChIP product. The dilution factor was then 20. Therefore, the % INPUT was calculated using the following formula:

% INPUT = $2^{(CqIN - Log2(DF) - CqCHIP)}$, where DF = 20. Cq^{IN} and Cq^{CHIP} are determined by qPCR (see above).

% of INPUT was calculated for anti-H3K27me3 ChIP product and for anti-H3 as a positive control to ensure the ChIP procedure worked.

3.13. Statistical analyses

All statistical analyses were performed on Graph pad Prism, using student t-test, Welch t- tests, two-way ANOVA, and significance of $P \leq 0.05$. All data are presented with mean +/- S.E.M. Tukey testing was used to test multiple comparisons in the ANOVA analyses. F test was used to compare variances.

Chapter 4: Results

4.1 H3K27me3 is detected at 17 kDA and 25 kDA in the skeletal muscle tissue

EZH2 mediated silencing mark H3K27me3 was of interest because MDM2 was seen to physically interact with the PRC2 complex subunit EZH2 (Wienken et al., 2016). H3K27me3 and EZH2 were usually measured in cancer cells (Duan et al., 2020). First, we used both whole skeletal muscle gastrocnemius pool from control C57BL6 female mice and C2C12 pool to test if we could detect H3K27me3 in muscle tissues and cells. Using 20 µg protein concentration from whole skeletal muscle and C2C12 pool, H3K27me3 was detected at 17 kDA (Figure 4.1). A surprising result was that there was an unexpected band at 25 kDA for both muscles and cells (Figure 4.1). This led us to test two more antibodies of H3K27me3 to ensure there was no non-specific binding occurring. H3K27me3 was detected by all three antibodies at both 17 kDA and 25 kDA in both muscle and C2C12 cells (Figure 4.1). This suggests that there could be certain post translational modifications occurring with tri-methylation on H3 lysine 27.

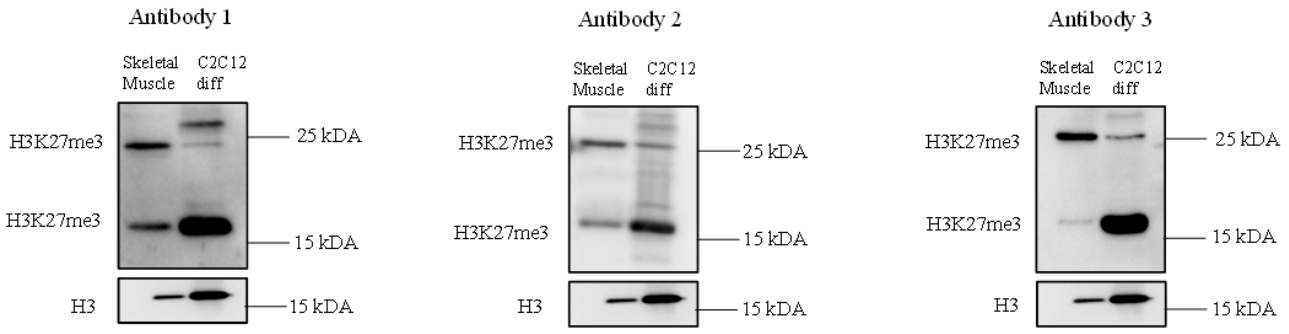
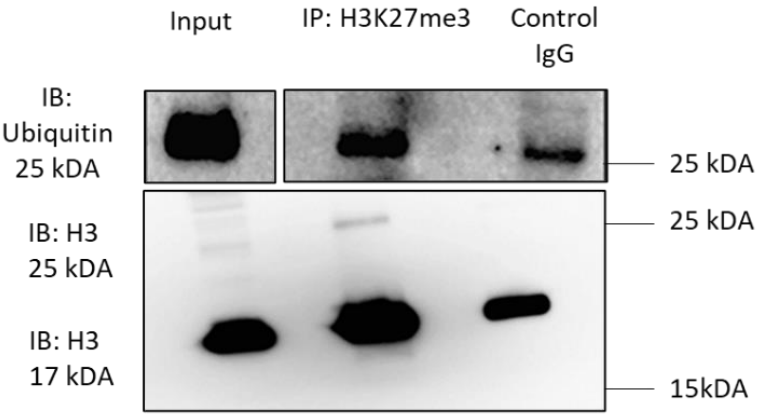


Figure 4.1: Protein levels of H3K27me3 show bands at 17 and 25 kDA in both skeletal muscle (gastrocnemius) from C57BL6 female mice and differentiated mouse C2C12 myotubes (C2C12 diff). Three different antibodies were used to detect H3K27me3 to confirm the specificity of the 25 kDA. Each antibody showed both a 17 kDA and a 25 kDA band for both cells and muscle. Antibodies used: antibody 1-Diagenode (C15410195, Burlington, Canada), antibody 2- Diagenode (C15410069, Burlington, Canada), and antibody 3- Cell signalling (C36B11, Whitby, Canada)

The next step was to investigate what the 25 kDA band could be. Indeed, NEDD4 is an E3 ubiquitin ligase, known to ubiquitinate and to stabilize MDM2 (Xu et al., 2015). Recently, NEDD4 was reported to

ubiquitinate H3 which caused the band to appear at the 25 kDA mark (Zhang et al., 2017). MDM2 is an E3 ubiquitin ligase, which means it can mono- or poly- ubiquitinate proteins, including histones H2A and H2B (Minsky & Oren, 2004). Additionally, MDM2 was observed to interact with the PRC2 complex, meaning it could potentially influence the EZH2 mediated histone mark H3K27me3 (Wienken et al., 2016). This led us to believe that the 25 kDA band could be a ubiquitinated form of H3. We immuno-precipitated 600 µg of whole skeletal muscle (control gastrocnemius) extract with anti-H3K27me3 antibody and then immuno-blotted for ubiquitin or H3. According to our immuno-precipitation (IP), we were able to detect H3 at 17 kDA, and a band at a higher molecular weight (25kDA) (Figure 4.2). Our negative control showed some non-specificity for the band detected at 17 kDA with our protocol (Figure 4.2). At 25 kDA, the control IgG did not lead to any band detection (Figure 4.2). This supports the notion that the band detected at 25 kDA is H3. While specific, the higher band is less abundant than the 17 kDA band (Figure 4.2). The densitometry of 17 kDA of H3 to its control IgG is about 2.0. When pulling down H3K27me3 and blotting for ubiquitin, a band was detected at the 25 kDA (Figure 4.2). Our negative control showed non-specificity detection for the 25 kDA band, however the densitometry of the 25 kDA band clearly indicated more ubiquitin being detected with pulling down H3K27me3 than the control rabbit IgG, with H3K27me3 to control IgG equal to 1.43 (Figure 4.2). This suggests that the observed 25 kDA could be a ubiquitinated form of H3 as it appeared more strongly in the IP compared to its control IgG.



	Densitometry signal	IP to Input ratio	IgG to Input ratio	Fold of change: IP compared to IgG control
Ubiquitin IP	**16128.62**	**0.2658**	**------**	**1.4267**
Ubiquitin Input	**60662.12**	**------**	**------**	
Ubiquitin IgG	**11303.74**	**------**	**0.1863**	
H3 25 kDA IP	**16226.17**	**16226.17/0**	**------**	**16226.17/0**
H3 25 kDA Input	**0**	**------**	**------**	
H3 25 kDA IgG	**0**	**------**	**0**	
H3 17 kDA IP	**72774.67**	**1.5321**	**------**	**2.0066**
H3 17 kDA Input	**47500.56**	**------**	**------**	
H3 17 kDA IgG	**36269.21**	**------**	**0.7635**	

Figure 4.2: Immunoblotting for H3 and Ubiquitin after immunoprecipitation of H3K27me3 shows detection of both Ubiquitin and H3 at a molecular weight of 25 kDA. Immuno-precipitation (IP) of H3K27me3 in 600 µg of control gastrocnemius muscle from C57BL6 female mice shows protein expressions of ubiquitin and H3 present at the 25 kDA band. Input was loaded at 20 µg concentration. Table shows IP to IgG ratio calculations based on densitometry signals from immunoblots.

4.2 Impact of fourteen days of endurance training on the acute response of histone modifications and histone modifiers to a bout of exercise in the gastrocnemius muscle

Next, we investigated the effect of exercise on MDM2 and the silencing mark H3K27me3. Since most literature studies exercise and activation marks, we decided to research more in depth the global abundance of the silencing mark H3K27me3 in the muscle epigenome to see if there is an overall enrichment of this silencing mark with exercise/training, independently of the location in the genome. The fourteen-day endurance training with an acute bout of exercise protocol aimed to test whether fourteen days of training could impact the acute response of certain histone modifications and histone modifiers to a bout of exercise.

First, we measured the proteins (referred to as histone modifiers) reported to regulate the establishment of these histone marks or the ubiquitination of H3: MDM2, EZH2, and its phosphorylated form on Threonine-311 (p-EZH2), and NEDD4 (Wienken et al 2016; Zhang et al., 2017; Minsky and Oren

2004). Next, we measured the global abundance of two histone modifications associated with gene silencing H3K27me3, H2AK199Ub, and one histone modification associated with gene activation, H3K4me3.

Using gastrocnemius protein extract, we investigated whether the protein levels of the E3 ubiquitin ligases capable of ubiquitinating histones, i.e., MDM2 and NEDD4, could change differently in response to an acute bout of exercise in sedentary and trained mice. We observed that MDM2 decreased by 39% in the 3-hour recovery after an acute bout of exercise only in the trained group, not in the sedentary group (Figure 4.3A & 4.3B). There were no significant changes detected in protein levels of NEDD4 with this acute exercise protocol in both sedentary and trained mice (Figure 4.3A & 4.3B). Since EZH2 catalyzes the establishment of H3K27me3, and the phosphorylation of EZH2 is associated with a lack of ability to catalyze H3K27me3 marks (Wan et al., 2018), we measured the protein levels of these histone modifiers. We observed no changes in the protein levels of EZH2 or p-EZH2 with fourteen-day training with an acute bout of exercise (Figure 4.3A & 4.3B.)

With regards to our histone modifications, we saw that H3K27me3 does not change at the 17 kDA band, however at the unexpected molecular weight of 25 kDA band we saw an overall decrease with acute bout of exercise (Figure 4.4A). Indeed, the two-way ANOVA analysis demonstrated an overall effect of the acute bout of exercise, independently of the training status ($P<0.01$). In the trained group, the level of the 25 kDA band significantly lowered by 54% after the 3 hours recovery period than before the bout of exercise. In the sedentary group, the acute bout of exercise had no significant impact of the levels of the 25 kDA bands detected. Acute exercise with or without recovery had no effect on the global abundance of H2AK119Ub and no effect on the global abundance of H3K4me3 in both sedentary and trained groups (Figure 4.4B & 4.4C).

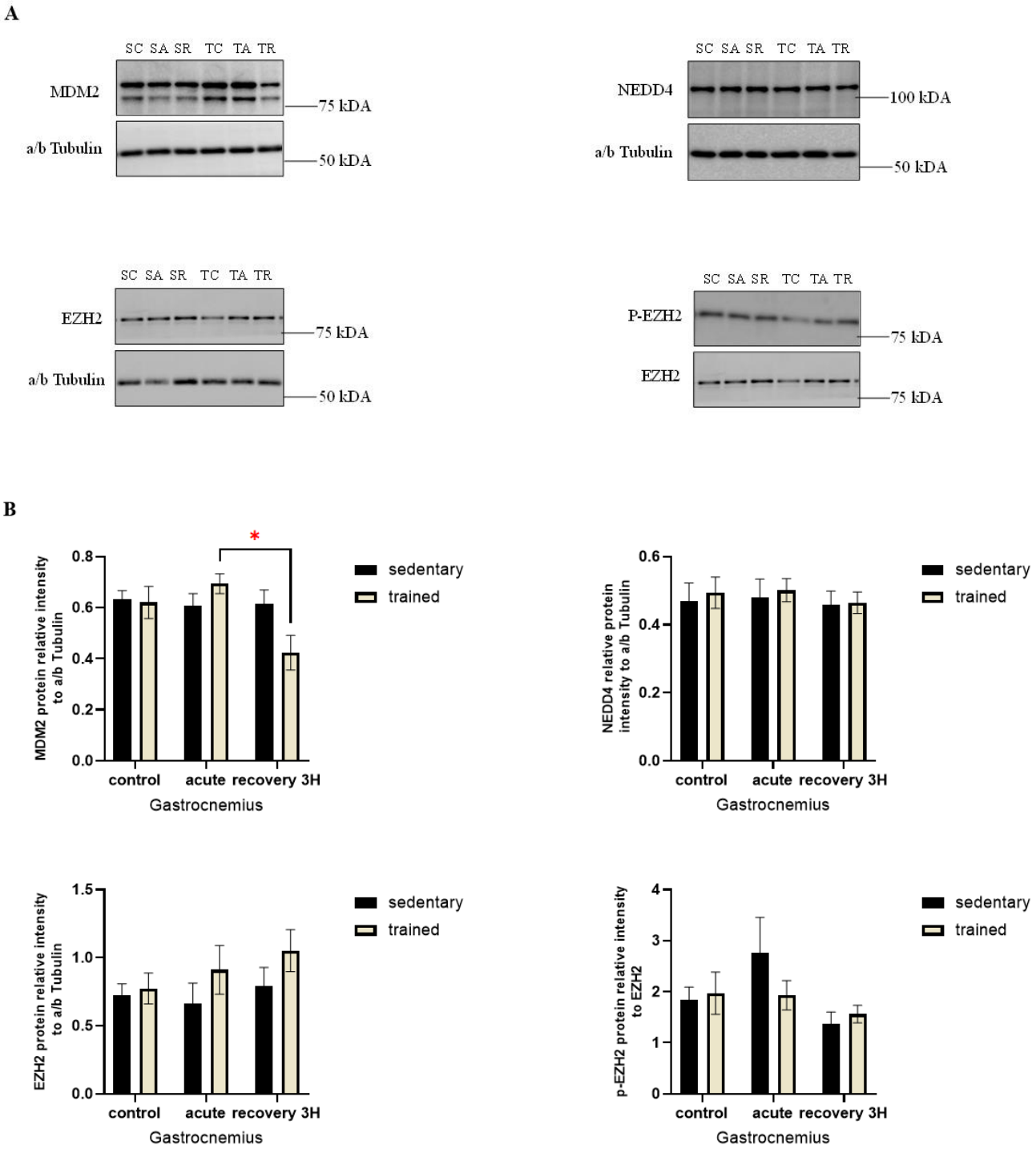


Figure 4.3: E3 ubiquitin ligase MDM2 decreases with 3 hours of recovery after an acute bout of exercise in 14 days trained mice. Effects of acute exercise protocol in sedentary and 14 days trained C57BL6 female mice gastrocnemius in protein expressions of MDM2, NEDD4, EZH2, and p-EZH2. (A) representative immunoblots for histone modifiers MDM2, EZH2, NEDD4, and p-EZH2. (B) two-way ANOVA for quantitative analyses for protein expressions and F-tests used to compare variances. Data shows mean +/- S.E.M., n=6. * Indicates significative differences $P<0.05$. Western blots are labelled as it follows: SC, sedentary control; SA, sedentary immediately post

acute bout of exercise; SR sedentary 3 hours post exercise (recovery); TC, 14 days training control; TA 14 training immediately post acute bout of exercise; TR, 14 days training 3 hours post acute bout of exercise (recovery).

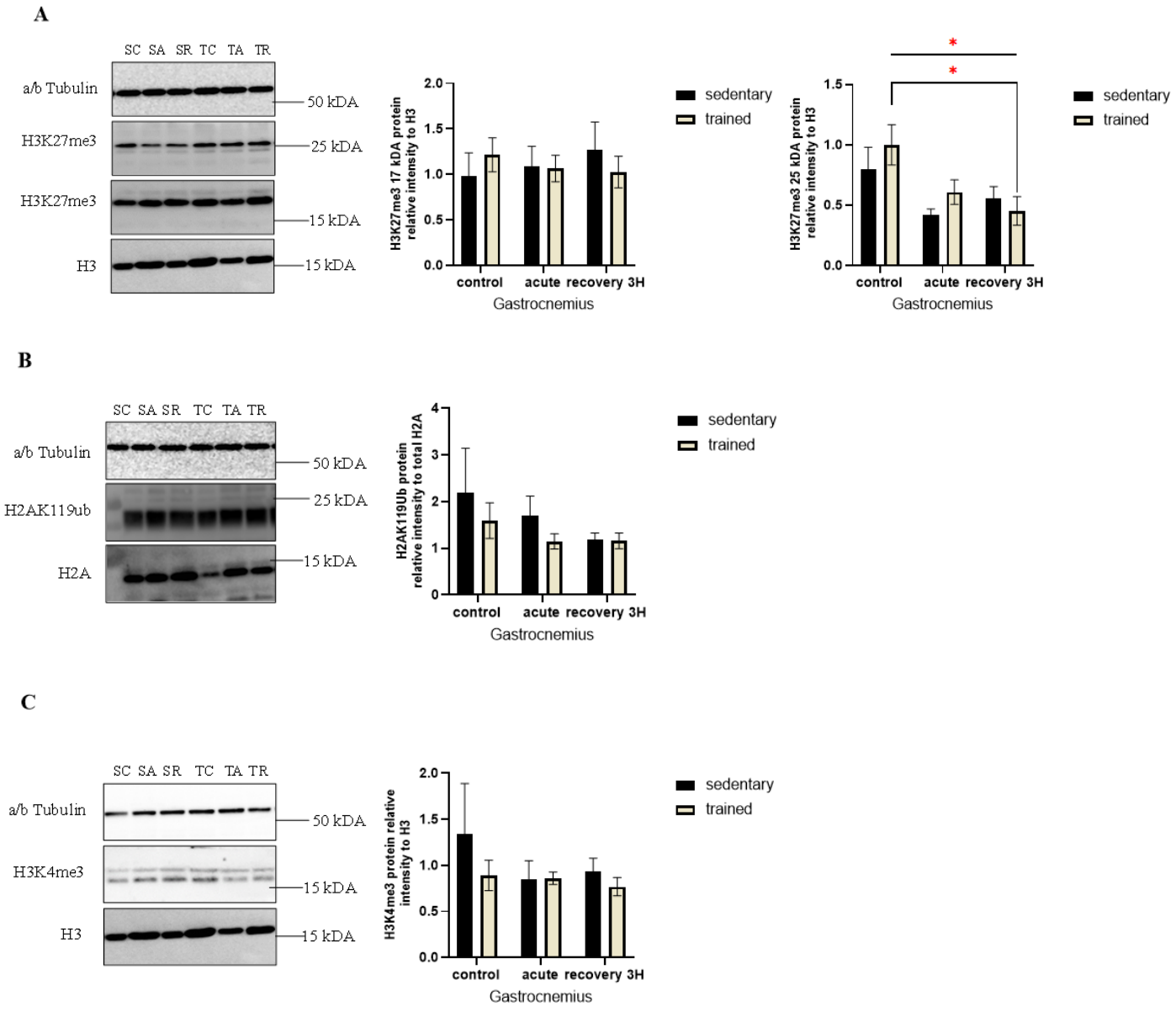


Figure 4.4: The overall effect of an acute bout of exercise with EZH2 mediated silencing mark H3K27me3 is the decrease at the unexpected molecular weight of 25 kDA. Effects of acute exercise protocol in sedentary and 14 days trained C57BL6 female mice gastrocnemius in protein expressions of H3K27me3 17 and 25 kDA, H3K4me3, and H2AK119ub. (A) representative immunoblots for histone modifications H3K27me3 and two-way ANOVA for quantitative analyses for protein expressions of H3K27me3 and F-tests used to compare variances. (B) representative immunoblots for histone modifications H2AK119ub and two-way ANOVA for quantitative analyses for protein expressions of H2AK119ub and F-tests used to compare variances. (C) representative immunoblots for histone modifications H3K4me3 and two-way ANOVA for quantitative analyses for protein expressions of H3K4me3 and F-

tests used to compare variances. Data shows mean +/- S.E.M., n=6. * Indicates significative differences P<0.05. Western blots are labelled as indicated in Figure 4.3.

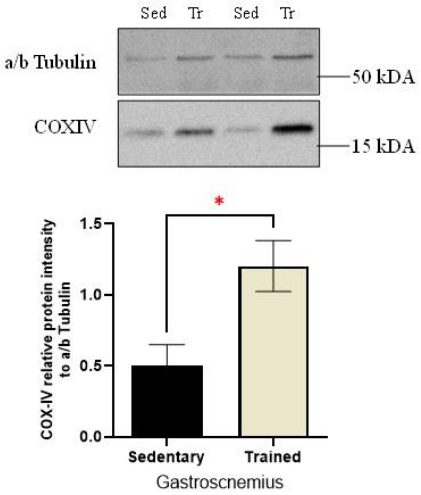
4.3 Impact of long-term endurance exercise on the resting level of histone modifications and associated histone modifiers in the gastrocnemius muscle

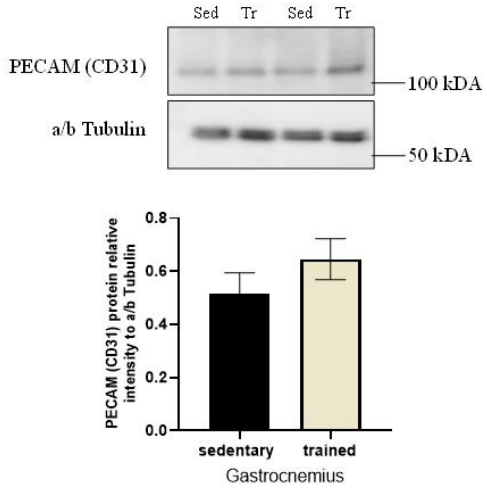
Since MDM2 was reported to increase with endurance training, as seen in the vastus lateralis muscle of humans after 6 weeks (Roudier et al., 2013), we questioned whether the resting levels of H3K27me3 would also be affected by 9 weeks of endurance training in the gastrocnemius of C57BL6 female mice.

We first verified that 9 weeks of endurance training promoted the expected skeletal muscle adaptations by measuring markers of oxidative metabolism (COX-IV) and of capillarization (PECAM/CD31) in the gastrocnemius muscle. The protein levels of PECAM (CD31) tended to increase (p=0.055) and COX-IV protein levels, as measured by a previous lab member (COX-IV, unpublished data), increased (Figure 4.5A). With regards to MDM2 and EZH2 in the gastrocnemius muscle, both protein levels increased in 9 weeks endurance trained mice were measured by a previous graduate student from the lab (Gulri, 2020) (Figure 4.5B). We next blotted for NEDD4, since NEDD4 overexpression increased H3 ubiquitination at the 25 kDA (Zhang et al., 2017). NEDD4 was seen to increase by 34% in response to 9 weeks of training (Figure 4.5B).

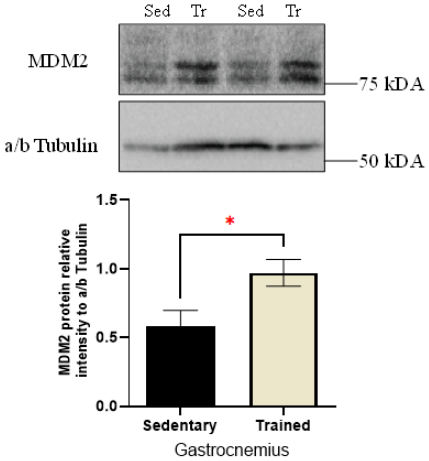
With 9 weeks of endurance training, the resting levels of gastrocnemius muscle showed that at the 17 kDA H3K27me3 protein expression did not change, while the protein expression at the 25 kDA band increased by 183% with training (Figure 4.6A). While we did not see any changes with the histone modification H3K4me3 (known for activation) (Figure 4.6B), we saw a surprising 36% decrease in the global protein level of the H3K9/14ac mark, which is associated with gene activation mark and regulated by NEDD4 (Zhang et al., 2017) (Figure 4.6C).

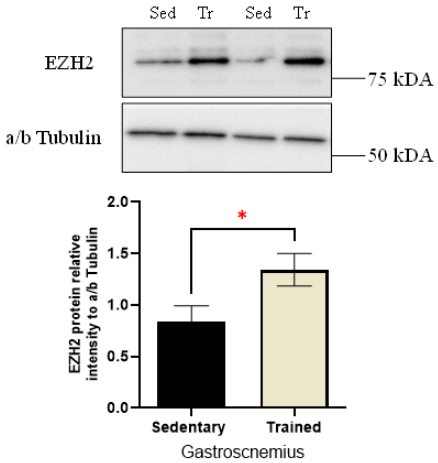
A


Sed Tr Sed Tr
a/b Tubulin
50 kDA
COXIV
15 kDA
COX-IV relative protein intensity to a/b Tubulin
1.5
1.0
0.5
0.0
*
Sedentary
Trained
Gastroscnemius


Sed Tr Sed Tr
PECAM (CD31)
100 kDA
a/b Tubulin
50 kDA
PECAM (CD31) protein relative intensity to a/b Tubulin
0.8
0.6
0.4
0.2
0.0
sedentary
trained
Gastrocnemius

B


Sed Tr Sed Tr
MDM2
75 kDA
a/b Tubulin
50 kDA
MDM2 protein relative intensity to a/b Tubulin
1.5
1.0
0.5
0.0
*
Sedentary
Trained
Gastrocnemius


Sed Tr Sed Tr
EZH2
75 kDA
a/b Tubulin
50 kDA
EZH2 protein relative intensity to a/b Tubulin
2.0
1.5
1.0
0.5
0.0
*
Sedentary
Trained
Gastroscnemius

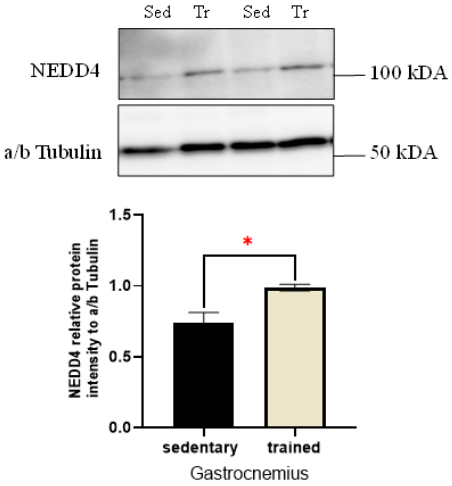

Sed Tr Sed Tr
NEDD4
100 kDA
a/b Tubulin
50 kDA
NEDD4 relative protein intensity to a/b Tubulin
1.5
1.0
0.5
0.0
*
sedentary
trained
Gastrocnemius

Figure 4.5: Increases in skeletal muscle adaptations with increases in resting level protein expressions of MDM2, EZH2, and NEDD4 in long term trained gastrocnemius. Effects of 9 weeks of endurance training in C57BL6 female mice gastrocnemius in resting levels (72 hours after last bout of exercise) protein expressions of PECAM (CD31), COXIV, MDM2, EZH2, and NEDD4. (A) representative immunoblots and quantitative analyses for protein expressions of COXIV (measured by previous lab member) and PECAM (CD31). Welch t-tests were used to assess differences. (B) representative immunoblots and quantitative analyses for protein expressions of MDM2 and EZH2 (measured by previous lab member), and NEDD4. Welch and student t-tests were used to assess differences. (A and B) Welch t-test were used when F-tests indicated that variances were different. Data shows mean +/- S.E.M., n=4-6 for gastrocnemius. * Indicates significative differences $P<0.05$.

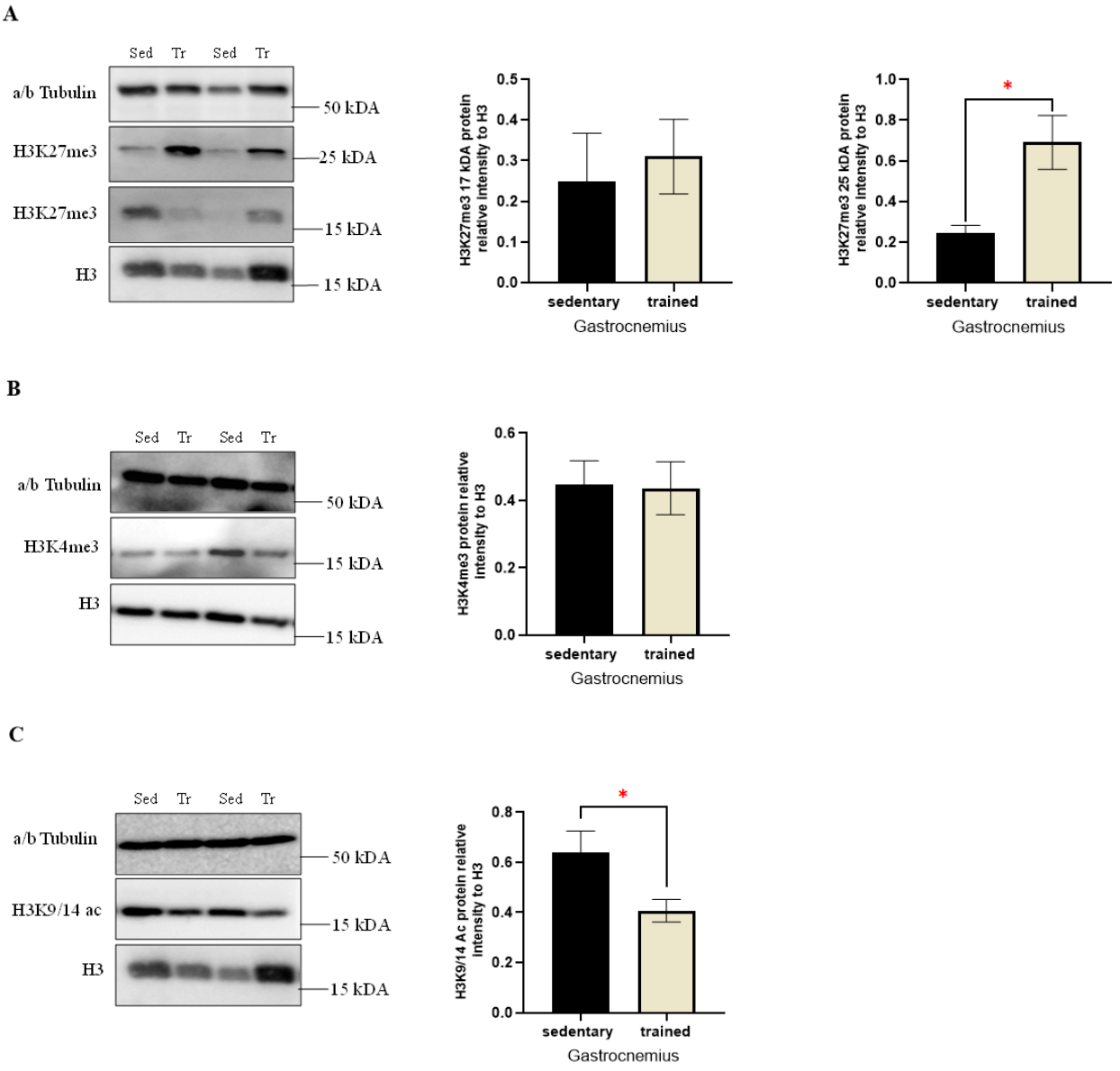


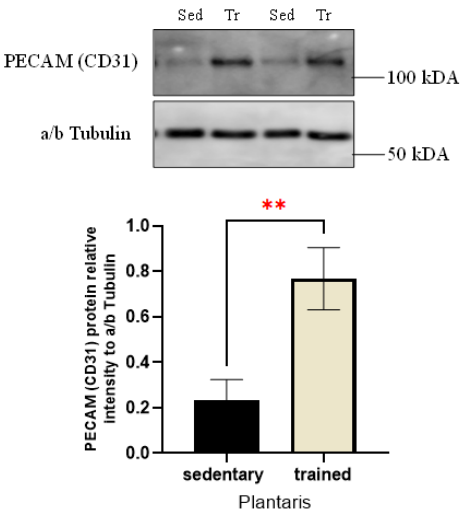
Figure 4.6: Resting levels of protein expression of EZH2 mediated silencing mark H3K27me3 does not change at 17 kDA but increases at unexpected molecular weight of 25 kDA, while histone modification H3K9/14ac decreases in long term trained gastrocnemius. Effects of 9 weeks of endurance training in C57BL6 female mice gastrocnemius in resting levels (72 hours after last bout of exercise) protein expressions of H3K27me3 17 and 25 kDA, H3K4me3, and H3K9/14ac. (A) representative immunoblots and quantitative analyses for protein expressions of H3K27me3 17 kDA and 25 kDA. (B) representative immunoblots and quantitative analyses for protein expressions of H3K4me3. (C) representative immunoblots and quantitative analyses for protein expressions of H3K9/14ac. F-tests used to compare variances. Student t-tests was used to assess differences. Data shows mean +/- S.E.M., n=4-6 for gastrocnemius. * Indicates significative differences $P<0.05$.

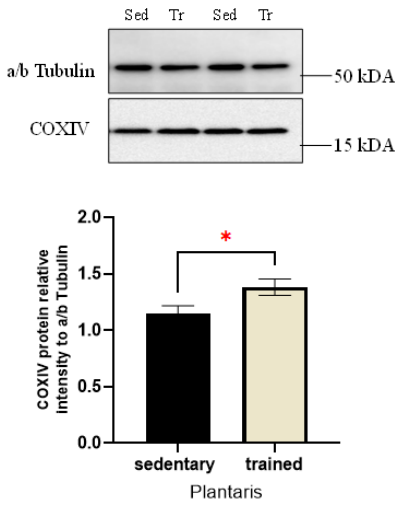
4.4 Impact of long-term endurance exercise on the resting level of histone modifications in the oxidative soleus and glycolytic plantaris skeletal muscles

We questioned if different muscle groups would have different expressions of the resting levels of H3K27me3 in regards to 9 weeks of training, so we further looked into the plantaris (glycolytic muscle) and the soleus (oxidative muscle). Verification of vascular and metabolic adaptations in the plantaris was done through measuring COXIV and PECAM (CD31). We observed that PECAM (CD31) and COXIV protein levels increased with training by 231% and 20% respectively (Figure 4.7A), verifying that adaptations to exercise occurred. No changes were observed in the protein levels of MDM2, EZH2, and p-EZH2 in the plantaris (Figure 4.7B).

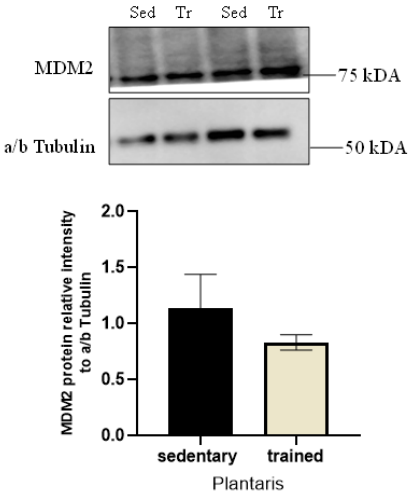
With regards to the histone silencing mark H3K27me3, we observed an 87% increase in the 25 kDA band detected with the anti-H3K27me3 antibody with 9 weeks of endurance training in the plantaris (Figure 4.8A); however, no changes to the histone activation mark H3K9/14 ac (Figure 4.8B). To further investigate the histone silencing mark H3K27me3, we measured the protein levels in the 9 weeks trained soleus muscle as well. In the soleus of C57BL6 female mice, there were no changes in protein expression detected at the 17 or the 25 kDA band of H3K27me3 (Figure 4.8C).

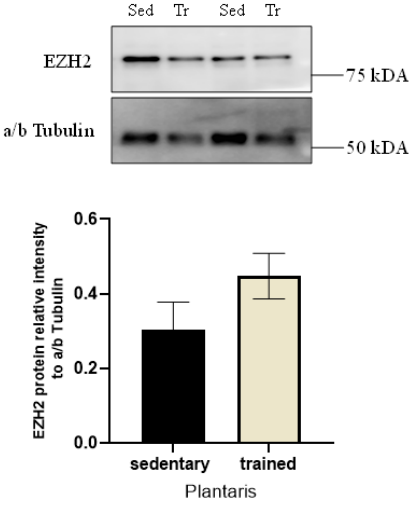
A


Sed Tr Sed Tr
PECAM (CD31)
100 kDA
a/b Tubulin
50 kDA
**
PECAM (CD31) protein relative Intensity to a/b Tubulin
1.0
0.8
0.6
0.4
0.2
0.0
sedentary
trained
Plantaris


Sed Tr Sed Tr
a/b Tubulin
50 kDA
COXIV
15 kDA
*
COXIV protein relative Intensity to a/b Tubulin
2.0
1.5
1.0
0.5
0.0
sedentary
trained
Plantaris

B


Sed Tr Sed Tr
MDM2
75 kDA
a/b Tubulin
50 kDA
MDM2 protein relative Intensity to a/b Tubulin
2.0
1.5
1.0
0.5
0.0
sedentary
trained
Plantaris


Sed Tr Sed Tr
EZH2
75 kDA
a/b Tubulin
50 kDA
EZH2 protein relative Intensity to a/b Tubulin
0.6
0.4
0.2
0.0
sedentary
trained
Plantaris

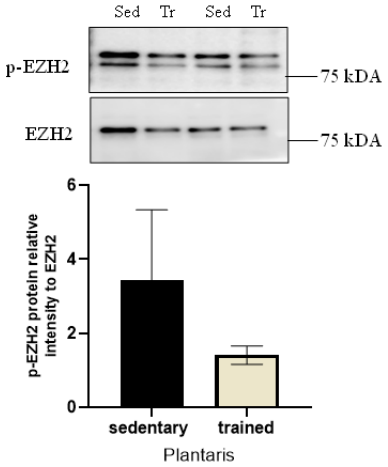
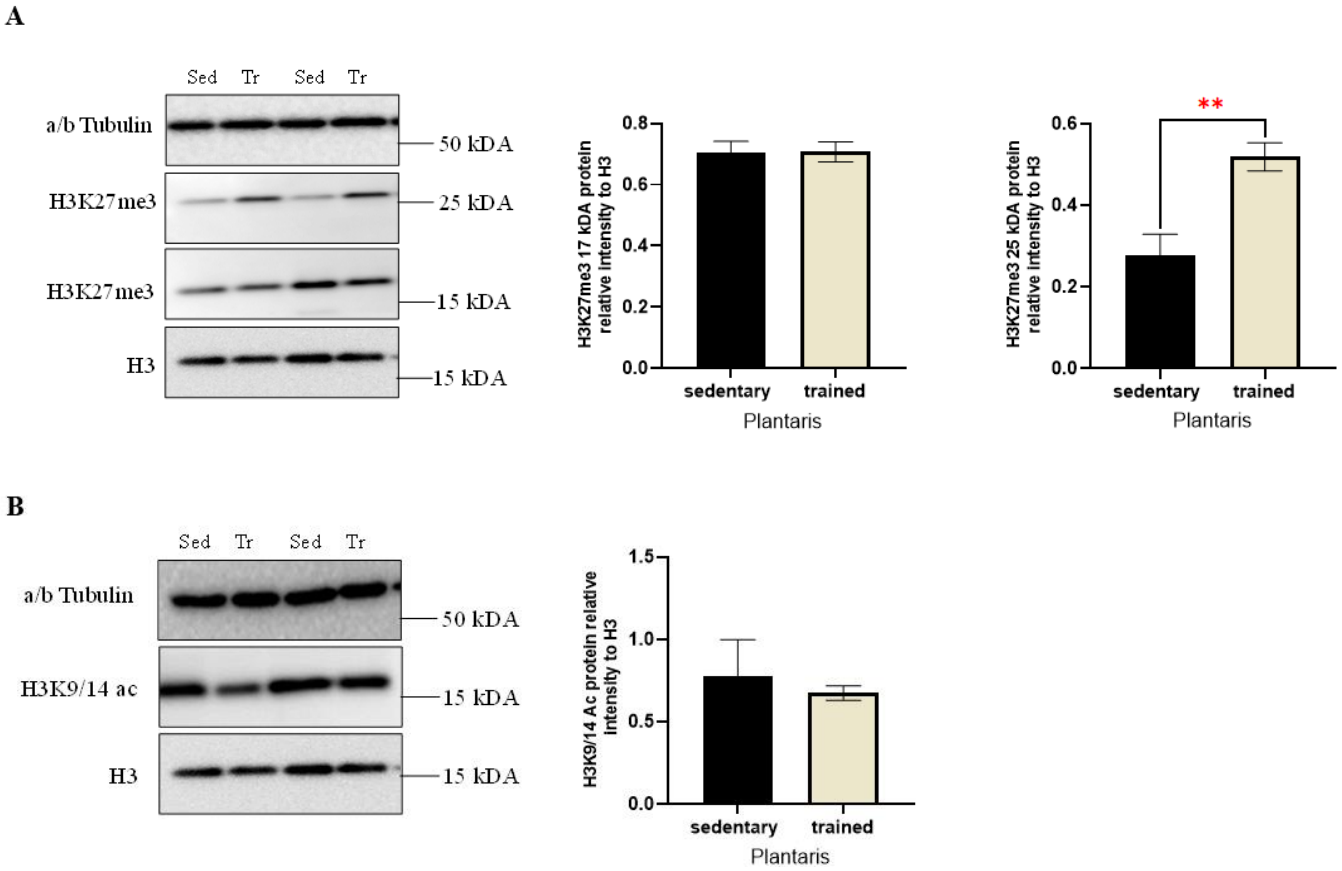

Sed Tr Sed Tr
p-EZH2
75 kDA
EZH2
75 kDA
p-EZH2 protein relative Intensity to EZH2
6
4
2
0
sedentary
trained
Plantaris

Figure 4.7: Increases in skeletal muscle adaptations with no increases in resting levels of protein expression of MDM2, EZH2, and p-EZH2 in the long term trained plantaris. Effects of 9 weeks of endurance training in C57BL6 female mice plantaris resting levels (72 hours after the last bout of exercise) protein expressions of COXIV, PECAM (CD31), MDM2, EZH2, and p-EZH2. (A) representative immunoblots and quantitative analyses for PECAM (CD31) and COXIV in the plantaris. Student t-tests was used to assess differences. (B) representative immunoblots and quantitative analyses for MDM2, EZH2, and p-EZH2 in the plantaris muscle. F-tests used to compare variances. Welch or student t-tests were used to assess differences. Data shows mean +/- S.E.M., n=5 or 6 for plantaris. * Indicates significative differences P<0.05.



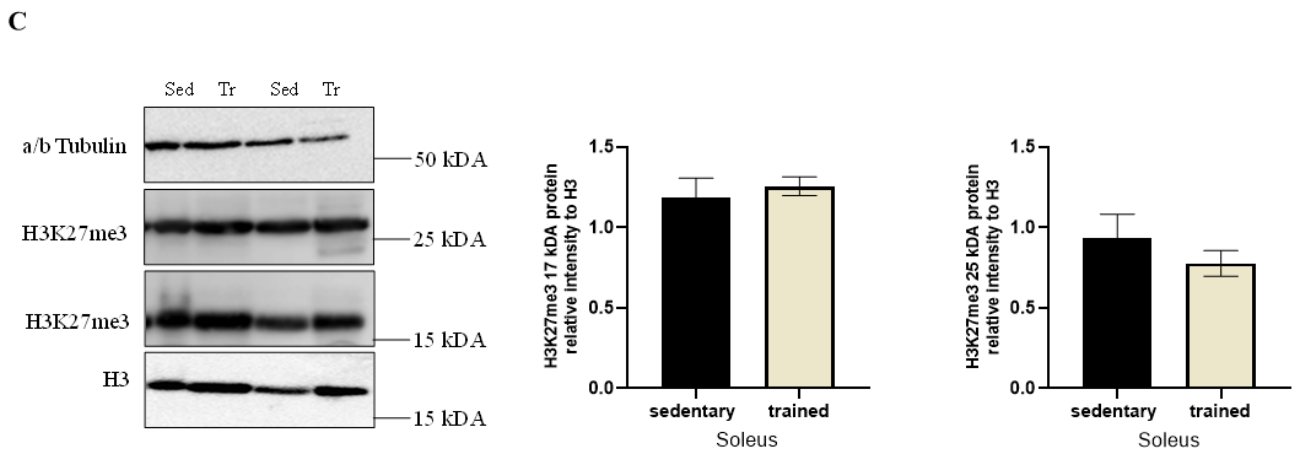


Figure 4.8: Resting levels protein expressions of histone modification H3K27me3 increases in 9 weeks trained plantaris with no changes in 9 weeks trained soleus. Effects of 9 weeks of endurance training in C57BL6 female mice plantaris resting levels (72 hours after the last bout of exercise) protein expressions of H3K27me3 17 kDA and 25 kDA, H3K9/14ac and in soleus resting levels (72 hours after the last bout of exercise) protein expressions of H3K27me3 17 kDA and 25 kDA. (A) representative immunoblots and quantitative analyses for H3K27me3 17 and 25 kDA in the plantaris. Variances were not different, student t-tests were used to assess differences. (B) representative immunoblots and quantitative analyses for H3K9/14ac in the plantaris. Variances were different between groups, Welch t-test was used to assess differences. (C) representative immunoblots and quantitative analyses for H3K27me3 17 and 25 kDA in the soleus. Variances were similar between groups, student t-test was used to assess differences. (A, B, C) F-tests used to compare variances. Data shows mean +/- S.E.M., n=5 or 6 for plantaris and soleus. * Indicates significative differences $P<0.05$.

4.5 Impact of long-term endurance exercise on one putative target genes of EZH2 and MDM2

We wanted to understand specifically the gene expressions that could be affected by 9 weeks of training in regard to gene silencing. Since we observed H3K27me3 at the 25 kDA to increase with 9 weeks of training in the gastrocnemius and the plantaris muscles, we wanted to know if we could see any changes in KDR gene expression. In a study by Wienken et al., MDM2 -/- MEFs had an increase in *Kdr* expression which was mirrored in EZH2-/- MEFs as well. Since MDM2 is known to increase with training, we wanted to know if skeletal muscles might show a relationship between MDM2, H3K27me3, and KDR. After 9 weeks of training, at resting levels of *Kdr* mRNA decreased by 44% in the gastrocnemius muscle of C57BL6 female mice (Figure 4.9A). We then looked at the plantaris to see if the *Kdr* mRNA expression

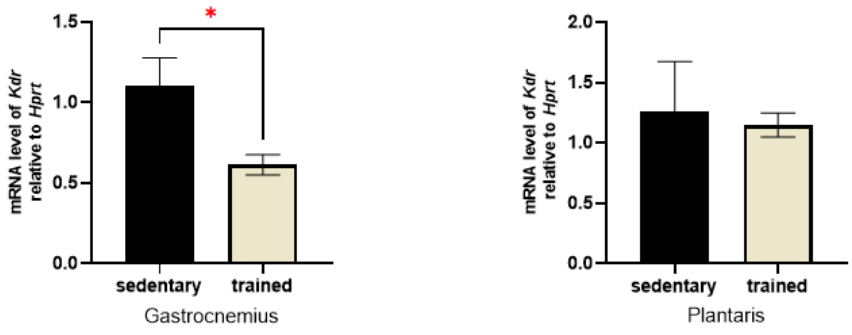
would also be affected, even though MDM2 and EZH2 both did not change with training. *Kdr* mRNA expression did not change with training in the plantaris (Figure 4.9A).

4.6 Impact of EZH2 inhibition in skeletal muscle endothelial cells.

Since KDR is highly expressed in ECs, we wanted to further investigate the expression of KDR in ECs in the skeletal muscle. For this, we specifically used mSMECs and inhibited EZH2 from the PRC2 complex, which would affect the histone silencing mark H3K27me3 (Shimizu & Kawano, 2022) using GSK343. With GSK343 treatment in mSMECs for 48 hours at a 1 µM concentration, H3K27me3 protein expression decreased by 30% and *Kdr* mRNA expression had increased by 22% (Figure 4.9B).

We next wanted to investigate if KDR and H3K27me3 had enhanced interactions at the KDR TSS with training which led to the decrease of the mRNA expression we saw in the gastrocnemius. Through ChIP, we pulled down the chromatin from sedentary and trained gastrocnemius of 9 weeks trained C57BL6 female mice using anti-H3K27me3 antibody and anti- H3 pan antibody for the control gastrocnemius muscle to serve as a positive control. Our qPCR results showed that on the *Kdr* gene, although there is no significance, there is a tendency for an increase in tri-methylation on histone 3 lysine 27 (Figure 4.10).

A



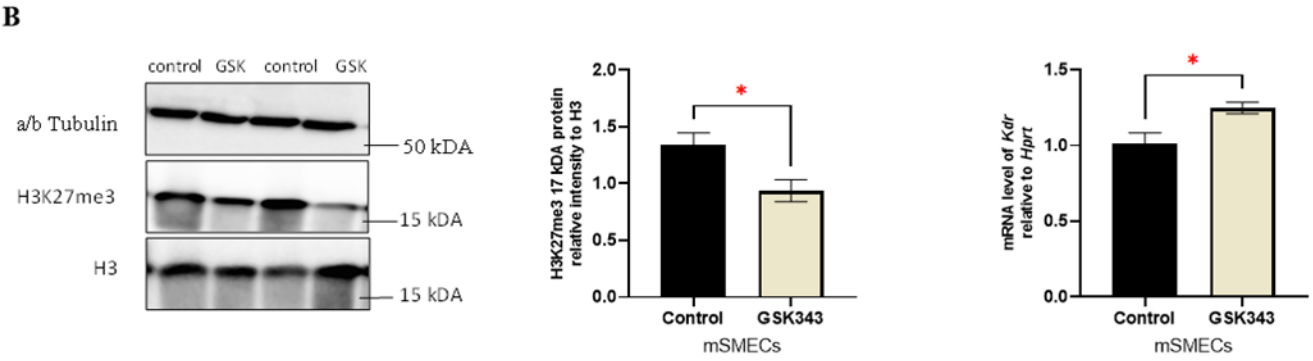


Figure 4.9: Resting levels of mRNA expression of angiogenic gene *Kdr* decreases with 9 weeks training in the gastrocnemius, but not in the plantaris. With EZH2 inhibitor GSK343, H3K27me3 protein expression decreased and *Kdr* mRNA levels increased in mSMECs. (A) Effects of 9 weeks of endurance training in C57BL6 female mice gastrocnemius and plantaris in resting levels (72 hours after the last bout of exercise) mRNA expression of angiogenic gene *Kdr*. Welch t-tests used for quantitative analyses for mRNA expressions and F-tests used to compare variances. Data show mean +/- S.E.M. n=7 for gastrocnemius and n=5 for plantaris. *Indicates significative differences P<0.05. (B) Effects of 48 hours of EZH2 inhibitor GSK343 (1 µM) treatment on mSMECs in protein expression of H3K27me3 at 17 kDA (left) and in mRNA expression of *Kdr* (right). Student t-tests used for quantitative analyses for both protein and mRNA expressions and F-tests used to compare variances. Data show mean +/- S.E.M., n=6 for mSMECs protein and n=4 for mSMECs mRNA. *Indicates significative differences P<0.05.

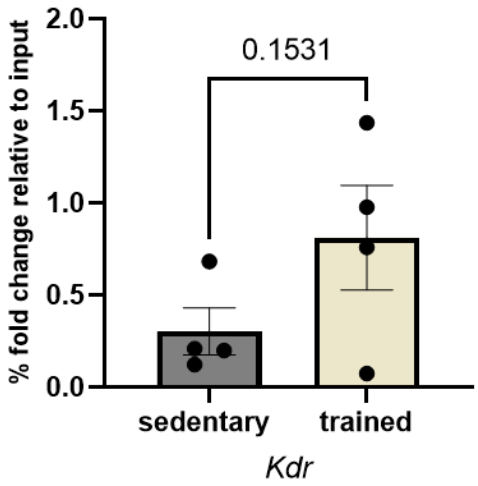


Figure 4.10: H3K27me3 has a tendency to be enhanced on the *Kdr* gene promoter with 9 weeks of endurance training in the gastrocnemius. Distribution of H3K27me3 silencing mark on the promoter of the gene *Kdr* was analysed in resting levels (72 hours after the last bout of exercise) of 9 weeks of endurance training C57BL6 female mice gastrocnemius. Chromatin immuno-precipitation (ChIP) done using H3K27me3 antibody, all samples were relative to their 20% inputs. Student t-tests used for quantitative analyses and F- tests used to compare variances for ChIP. Data shows mean +/- S.E.M., n=4 for gastrocnemius.

Chapter 5: Discussion

The focus of this thesis was to investigate the silencing mark H3K27me3 in the context of different exercise protocols. As highlighted above, epigenetics in the context of exercise has yet to analyze in depth the role of histone silencing marks. Angiogenesis and capillary formation are studied extensively as well; however, the mechanisms behind capillary stabilization after 4 weeks of continuous exercise at the same intensity and duration has not been clearly understood (Haas & Nwadozi, 2015). MDM2, a protein that has pro-angiogenic functions and plays a role in epigenetic functions, has been seen to physically interact with the PRC2 complex subunit EZH2 (Wienken et al., 2016). The PRC2 complex is a known HMT which is famously known for establishing the H3K27me3 silencing mark. The most surprising and novel finding in this thesis is that H3K27me3 was observed at two separate bands, one at 17 kDA and the other at 25 kDA in both skeletal muscle and C2C12 cells. H3K27me3 is a known histone mark at the 17 kDA, which is what we observed in our mSMECs protein expressions. Since we did not observe a 25 kDA band in our mSMECs, we wonder if the 25 kDA band of H3K27me3 is specific to skeletal muscle cells. Our IP in control gastrocnemius of C57BL6 female mice, showed that the 25 kDA band could be a ubiquitinated form of H3. Although, the IgG control for our IP showed some non-specificity, the intensity of our IP was much stronger than the IgG. The likely reason for our IgG control showing non-specificity could be due to our protocol not being efficient in pulling down endogenous IgG from skeletal muscle extract. Future steps would be to optimize our IP protocol to ensure that it can work well with skeletal muscle extracts, not just cells.

Another interesting finding in this thesis is the protein changes of the 25 kDA band (not the 17 kDA) of H3K27me3 with exercise and training. With the acute exercise protocol in C57BL6 female mice in the gastrocnemius, the H3K27me3 protein levels had an overall decrease with a 1-hour acute bout of exercise compared to the control trained and sedentary mice. This trend of decrease was mirrored in MDM2; MDM2 protein levels were observed to decrease with 3-hour recovery after an acute bout of exercise. It is interesting to note that MDM2, as a pro-angiogenic molecule, is decreasing after recovery from an acute bout of exercise in the 14 day trained mice, when in theory, we would expect to see an increase since the

14 days is the peak window of capillary formation/angiogenesis (Haas & Nwadozi, 2015). Even though MDM2 protein levels did not increase with 1 bout of exercise in human, rats, or mice, MDM2 activity increased through the increase of phosphorylation of MDM2 on serine 166 (Aiken et al., 2016). The increase in phosphorylated MDM2 can indeed support a pro-angiogenic pathway in ECs as it led to the increase in MDM2 activity on FOXO1 (Aiken et al., 2016). The protein levels of MDM2 increases with prolonged exercise training in rat plantaris muscles with an increase in capillarization as well (Aiken et al., 2016; Roudier et al., 2012). The next step could be to measure the protein levels of phosphorylated MDM2 on serine 166 in the acute exercise protocol to help understand the decrease of MDM2 protein expression we see; since phosphorylation of MDM2 on serine 166 has been observed to decrease the chromatin binding of MDM2 (Riscal et al.,2016), there could be a potential decrease in phosphorylated MDM2 on serine 166 which could increase MDM2's binding to the chromatin. The decrease in H3K27me3 silencing mark fits in the idea that there are less silencing occurring after 14 days of performing daily endurance exercise. This question whether this decrease in H3K27me3 is required for or capillary formation to support skeletal muscle angio-adaptation.

With VEGF-A stimulated primary ECs, H3K4me3 and H3K27me3 were seen to be bivalent histone marks facilitating transcriptional regulation of certain transcription factors (Kanki et al., 2022). The PRC1 mediated histone mark H2AK119ub had reduced due to the H3K4me3 marks fifteen minutes after VEGF-A stimulation in ECs (Kanki et al., 2022). Along with this, H3K27me3 marks were present at all time frames of VEGF-A stimulation on those transcription factors (Kanki et al., 2022). The PRC2 complex can interact with PRC1 complex which is in charge of ubiquitinating H2AK119, which supports the establishment of H3K27me3 marks at PcG target genes (Uckelmann & Davidovich, 2021). H3K9/14 ac marks were seen to increase in response to NEDD4 and H3 ubiquitination (discussed later in depth). Thus, we decided to measure H3K4me3, H2AK199ub, and H3K9/14 ac. Even though H2AK199ub and the activation marks H3K4me3 and H3K9/14 ac that we measured did not change with the acute exercise protocol, it could suggest that with the 14-day acute exercise protocol, these activation marks or other

activation marks could be more pronounced in an earlier or later stage of angio-adaptation. For instance, acetylation of H3 was seen to increase after 4 weeks of treadmill running for 60 minutes/day in rats (Ohsawa et al., 2018) and H3K36 acetylation increased after 60 minutes of cycling in humans (McGee et al., 2009).

With 9 weeks of training in C57BL6 female mice, we saw the increase in the protein expressions of H3K27me3 at the 25 kDA in both the gastrocnemius and the plantaris, with a stronger increase noted in the plantaris. MDM2 protein is most expressed in muscles that have higher oxidation and greater capillarization, such as the heart and soleus, compared to muscles like EDL and plantaris (Roudier et al., 2012). This could help explain the non significance seen in MDM2 protein levels after 9 weeks of endurance training in the plantaris, even though there is a strong increase in H3K27me3 at the 25 kDA band with training, which could be linked to capillarization (discussed further below).

MDM2 was observed to increase with 6 weeks of endurance training in humans, which was followed by an increase in PECAM (CD31) that shows that capillarization was achieved (Roudier et al., 2013). The lab has also observed MDM2, along with EZH2, to increase with 9 weeks of endurance training in the gastrocnemius of C57BL6 female mice (unpublished data from previous master student). Going further with these observations, two proteins of interest were NEDD4 and H3K9/14 ac. NEDD4 physically interacts with MDM2 at its RING domain (where ubiquitination occurs) and NEDD4 knockout in MEFs showed a decreased ability to ubiquitinate MDM2 (Xu et al., 2015). With glucose, H3 was ubiquitinated through NEDD4, showing up at the 25 kDA band (Zhang et al., 2017). This also led to the induction of acetylation marks on H3 on K9 and K14, which were also dependent on NEDD4 (Zhang et al., 2017). In the gastrocnemius we observed NEDD4 increasing while H3K9/14 ac decreasing. This observation was an interesting one because it solidifies the idea that with the increase of H3K27me3 silencing mark at the 25 kDA band with 9 weeks of training, the activation mark H3K9/14 ac decreased, meaning that silencing marks are more pronounced rather than activation marks with long term training.

The mRNA levels of VEGFR2/KDR were measured in both gastrocnemius and plantaris muscle from 9 weeks trained C57BL6 female mice. Our interest in KDR peaked after the study by Wienken et al,

showing that in MDM2-/- MEFs, *Kdr* mRNA expression increased, which was also seen with EZH2 -/- MEFs (Wienken et al, 2016). KDR is known to be highly expressed in ECs; however, mRNA levels of KDR have been studied extensively in different muscle models. Here we found that *Kdr* decreased with training in the gastrocnemius while in the plantaris it was non significant. One reason why we could be observing this is because the plantaris being a glycolytic muscle, is known to achieve adaptations to exercise more easily than mixed or oxidative muscles (Birot et al., 2003; Egginton, 2008; Hudlicka et al., 1992). This was also backed up by our findings of PECAM (CD31) and COXIV protein expressions being increased significantly in the plantaris, while in the gastrocnemius even though it was non significant, it had a tendency to increase (P=0.055). This could be due to the cut of the gastrocnemius muscle during protein extraction, since the gastrocnemius is a mixed fiber type muscle, with both glycolytic and oxidative fibers, the cutting of the muscle could have been insufficient in acquiring a muscle section that would reflect the proper capillarization achieved from training. This could suggest that the angio-adaptation in the plantaris has not yet reached capillary stabilization as much as the gastrocnemius did after 9 weeks of training, even though both muscles had increases in H3K27me3 marks with training.

Interestingly, other studies observed different mRNA levels of KDR. With just 1 bout of treadmill exercise in rats' mRNA levels of *Kdr* did not increase, while after 1 hour of cycling in humans, *KDR* mRNA increased by 5 folds 4 hours postexercise (Birot et al., 2003; Gavin et al., 2004). Similar results were seen with resistance exercise, where *KDR* mRNA levels were increased 4 hours postexercise (Gavin et al., 2007). The KDR protein levels were doubled from basal level in muscles after two days of electrical stimulus and increased by 50% after 4 days in rat EDL (Milkiewicz et al., 2003). In both normoxia and chronic hypoxia conditions, rat gastrocnemius were observed to have decreased mRNA levels of *Kdr* after an acute bout of exercise in both sedentary and 8 weeks trained rats (Olfert et al., 2001). The potential reason for this could be due to the difference in the time of muscle extraction from the last bout of exercise. Our 9 weeks trained muscles had a 72-hour washout period, considered resting levels, before muscle extraction, this can affect the level of expressions of mRNA. There could be a difference in mRNA expressions of genes when

measuring immediate postexercise compared to resting levels after the last bout of exercise. Many genes can be down regulated after returning to resting levels after exercises, which could also explain the decrease in *Kdr* mRNA levels in the gastrocnemius.

According to the research done by Shimizu and Kawano in mice tibialis anterior muscle, acute exercise decreased the overall abundance of H3K27me3 two hours postexercise (Shimizu & Kawano, 2022), which was also similarly reflected in our acute exercise data. With mice administered EZH2 inhibitor GSK343 thirty minutes prior to their acute bout of exercise, GSK343 treated sedentary mice showed a decrease in H3K27me3 levels, and GSK343 treatment increased the abundance of H3K27me3 on genes like PGC-1α post two hours of exercise (Shimizu & Kawano, 2022). This was a surprising finding, since GSK343 is an inhibitor of EZH2, it would assumingly also inhibit the tri-methylation of H3K27. To understand the role of KDR and H3K27me3 in ECs specifically, we decided to look at mSMECs and treated them for 48 hours with the EZH2 inhibitor GSK343. We noticed that the protein levels of H3K27me3 decreased with the inhibition of EZH2 which was expected; however, the increase in *Kdr* was exciting as it suggests that KDR could indeed be affected by the silencing of the PRC2 complex.

The chromatin immuno-precipitation assay is an assay that studies the protein DNA interaction to give information on the chromatin state and gene transcription (extracted from Cell signalling chromatin immuno-precipitation protocol, Solomon et al., 2021). Here we investigated the interactions between histone modification H3K27me3 and the *Kdr* TSS region to understand if there are more silencing marks placed on *Kdr* in the 9 weeks trained gastrocnemius muscle. Since we saw that in the 9 weeks trained gastrocnemius muscles, *Kdr* mRNA decreased, we wanted to measure the H3K27me3 marks on the *Kdr* gene itself through the ChIP-qPCR. We normalized our CT values through the most used methods in ChIP-qPCR, which is the fold enrichment method which represents the signal over background noise (Solomon et al., 2021). The fold enrichment method uses the ratio of the amount of target sequence in the IP lysate to the amount in input lysate (Solomon et al., 2021). Even though we did not see significance, we saw that there was a tendency for higher H3K27me3 marks on *Kdr* in the 9 weeks trained than in the sedentary. One

reason for not seeing significance could be due to insufficient chromatin extraction from the skeletal muscle resulting in a lower yield of chromatin. This could offset some samples in the quality and quantity of the chromatin. Future steps would be to optimize the ChIP protocol to ensure better chromatin extraction from muscles and to use more muscle samples.

The future studies that would help in gaining more insight in relation to this thesis would be to do an RT-qPCR to measure the mRNA levels of *Kdr* in the acute exercise protocol as well as the protein levels of phosphorylation of MDM2 on serine166. This could help us understand the effect of the acute exercise protocol with regards to the decrease of MDM2 and H3K27me3 noted and the *Kdr* gene. It would also give us another time point in assessing mRNA since the acute exercise protocol muscles were collected immediately or 3 hours after the last bout of acute exercise. With regards to cell culture, the next step would be to repeat the mSMECs 48-hour GSK343 treatment to ensure that the preliminary data was reproducible. Another experiment would be to inhibit MDM2 in mSMECs to investigate if KDR and H3K27me3 have similar results as with EZH2 inhibition. Another interesting experiment would be to over express MDM2 and EZH2 in mSMECs to see effects on H3K27me3, KDR, and NEDD4. Finally, to further investigate the function of H3 ubiquitination in muscles and angiogenic gene expression would be of interest.

In summary, H3K27me3 was newly founded at the 25 kDA band in both skeletal muscle and C2C12 cells, with the 17 kDA band of H3K27me3 only observed in the mSMECs, suggesting that the 25 kDA band is a muscle cell specific band that is affected by different exercise protocols. The pro-angiogenic protein MDM2 seems to follow the trends of the 25 kDA band of H3K27me3 with different exercise protocols in the gastrocnemius. Further investigation led us to believe that the 25 kDA band of H3K27me3 could be an ubiquitinated form of this histone. Both gastrocnemius and plantaris muscles were observed to have an increase in H3K27me3 at the 25 kDA band with 9 weeks of training, however the plantaris did not show any significant changes in MDM2 and EZH2 protein levels and in *Kdr* mRNA expressions. The 9 weeks trained gastrocnemius muscle showed significant decrease in the *Kdr* mRNA expression, while

mSMECs treated with an EZH2 inhibitor showed an increase in *Kdr* mRNA levels. ChIP data reveals that there could be a potential for more H3K27me3 marks to be present on the trained *KDR* gene.

The data fits well with our hypothesis that MDM2 works together with the PRC2 complex EZH2 subunit to regulate the histone silencing mark H3K27me3 to regulate certain angiogenic genes. The increase of NEDD4 with training could suggest an interaction with MDM2. Since NEDD4 is seen to stabilize MDM2 through ubiquitination, this could suggest that NEDD4 is stabilizing MDM2 with 9 weeks of training, which could be strengthening the interaction of MDM2 and EZH2, in turn increasing the H3K27me3 mark on target genes. This pathway could increase silencing on different genes, in this case KDR, which is why its mRNA levels decrease with 9 weeks of training (Figure 5.1A). With regards to the mSMECs, since it is a type of ECs, MDM2 and EZH2 could interact together to regulate H3K27me3 at the 17 kDA to affect the mRNA expression of KDR (Figure 5.1B). Additionally, our data questions whether the increase in NEDD4 induced by endurance exercise supports the skeletal muscle adaptation through H3 modifications. Indeed, NEDD4 was reported to promote H3 ubiquitination allowing for reactivation of genes via H3 ubiquitination (Zhang et al., 2017) and to regulate the activity of PRC2 in muscle cells under oxidative stress (Liu et al., 2019). This led us to believe that our newly identified 25 kDA band of H3K27me3 in the skeletal muscle could be a ubiquitination of H3 through the histone modifier NEDD4.

Our research investigated the global abundance of different histone modifications and histone modifiers in the whole skeletal muscle with different exercise protocols, and the local abundance of H3K27me3 in skeletal muscle microvascular endothelial cells with EZH2 inhibition. Our thesis discovered the increase in H3K27me3 silencing marks after 9 weeks training and a decrease after acute exercise that was more pronounced after short-term training. This can suggest the role of H3K27me3 silencing marks in regulating skeletal muscle adaptation and potentially angio-adaptation, as we observed increased tendency of H3K27me3 marks on TSS of *Kdr* in 9 weeks trained gastrocnemius muscle. MDM2 and EZH2 are both proteins studied extensively in the context of cancer and tumour angiogenesis, their roles in skeletal muscle angiogenesis are yet to be explored in depth. Studying the interactions between MDM2, EZH2, and the

related histone silencing mark H3K27me3, can bring new insight in the mechanisms regulating angio-adaptation. This can help find new ways to maximize and to potentially facilitate capillary formation before stabilization

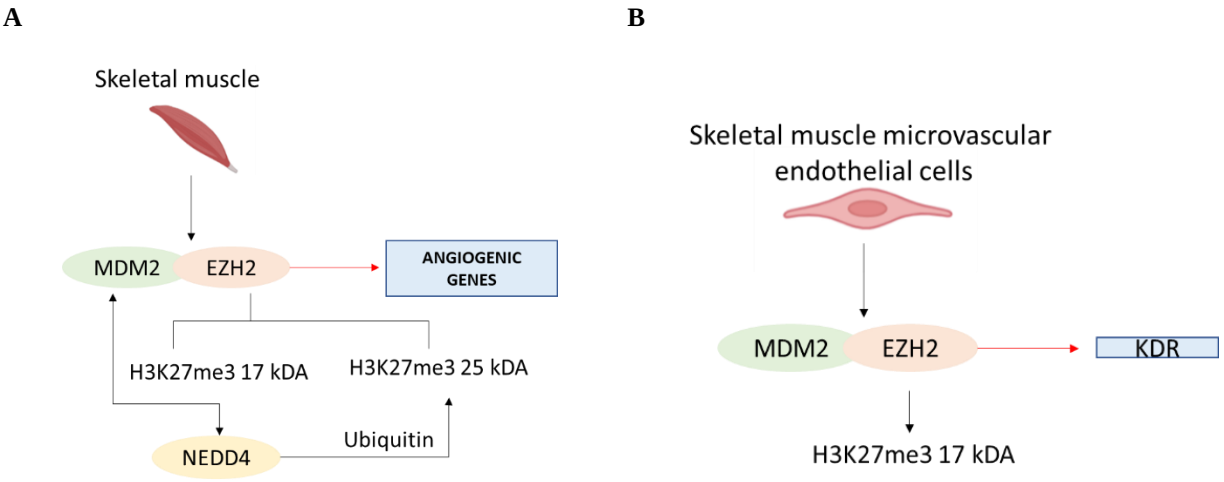
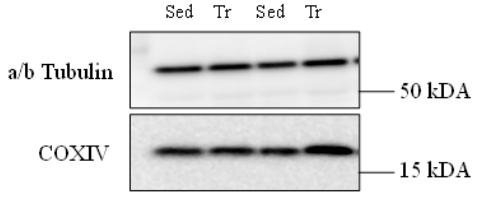


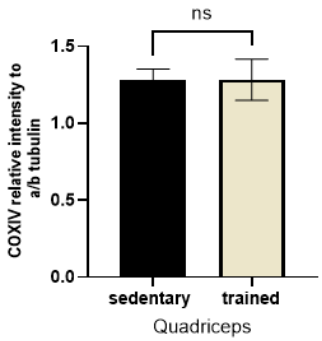
Figure 5.1: Schematic diagrams of potential pathway interactions of MDM2 and EZH2 with regards to the histone silencing mark H3K27me3 in the skeletal muscle (A) and in the ECs (B) in regulating angiogenic gene KDR. Created on Biorender.

Chapter 6: Supplemental Data

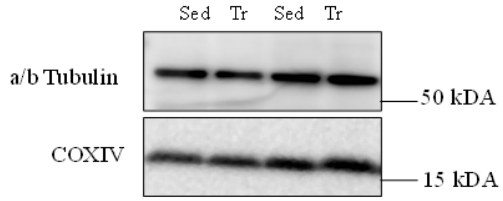
A second set of mice were trained exactly as indicated in the methods for long-term training. The skeletal muscle adaptations were not observed as expected. We suspect that the quality of the muscles deteriorated due to the break down of the -80°C freezer, causing thawing and re-freezing cycle. For this reason, this second set of 9 weeks trained C57BL6 female mice muscle samples were not included into our analyses.

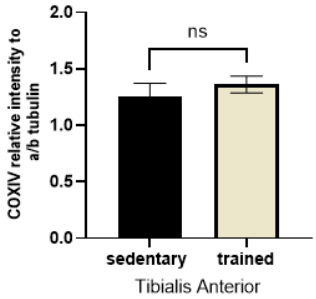
A



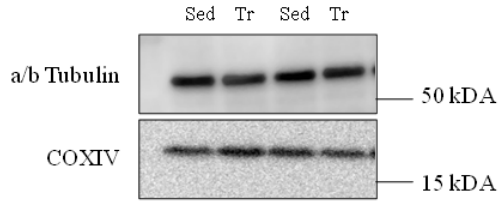


B





C



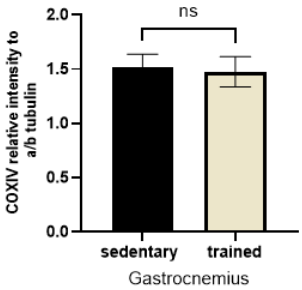
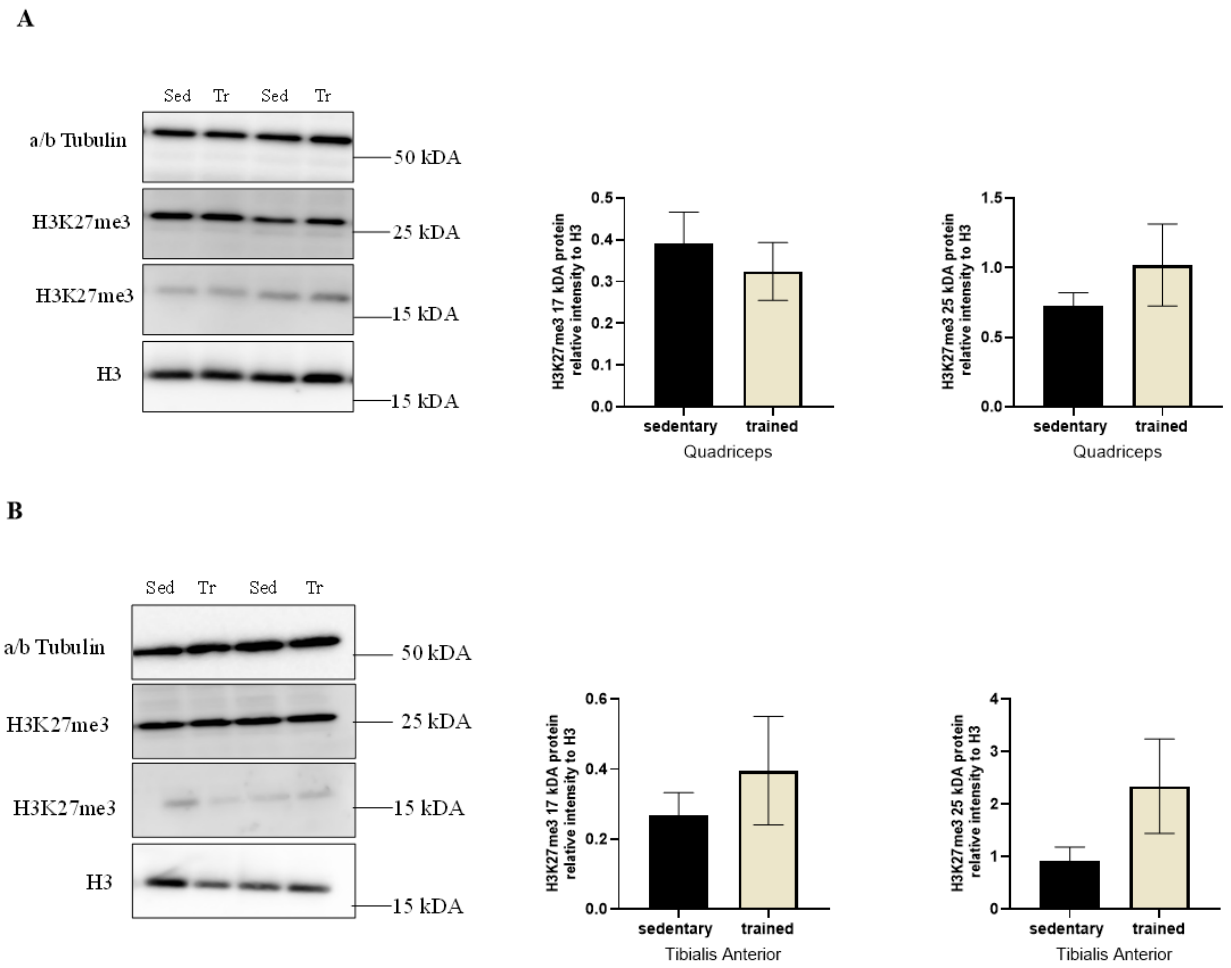


Figure 6.1: No changes in resting levels of protein expression of COXIV in 9 weeks trained quadriceps, tibialis anterior, and gastrocnemius muscles of second set C57BL6 female mice. Effects of 9 weeks of endurance training in second set C57BL6 female mice muscles. Resting levels of protein expression of COXIV were measured. (A) representative immunoblots and t-tests for quantitative analyses for quadriceps (B) representative immunoblots and t-tests for quantitative analyses for tibialis anterior (C) representative immunoblots and t-tests for quantitative analyses for gastrocnemius. Data shows mean +/- S.E.M., n=5, * Indicates significative differences P<0.05.



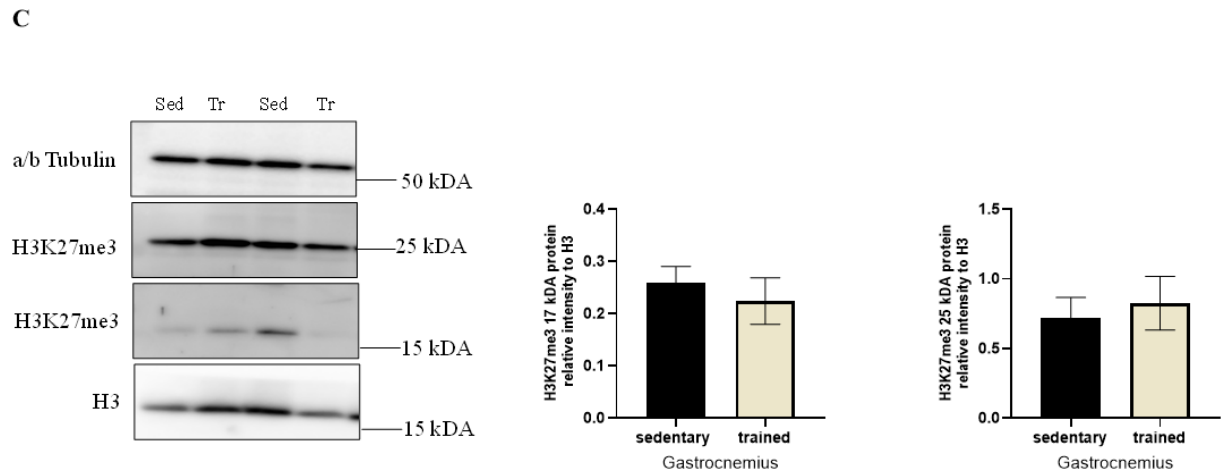


Figure 6.2: No changes in resting levels of protein expression of H3K27me3 in 9 weeks trained quadriceps, tibialis anterior, and gastrocnemius muscles of second set C57BL6 female mice. Effects of 9 weeks of endurance training in second set C57BL6 female mice muscles. Resting levels of protein expression of H3K27me3 at 17 kDA and 25 kDA were measured. (A) representative immunoblots and t-tests for quantitative analyses for quadriceps (B) representative immunoblots and t-tests for quantitative analyses for tibialis anterior (C) representative immunoblots and t-tests for quantitative analyses for gastrocnemius. Data shows mean +/- S.E.M., n=5, * Indicates significative differences P<0.05.

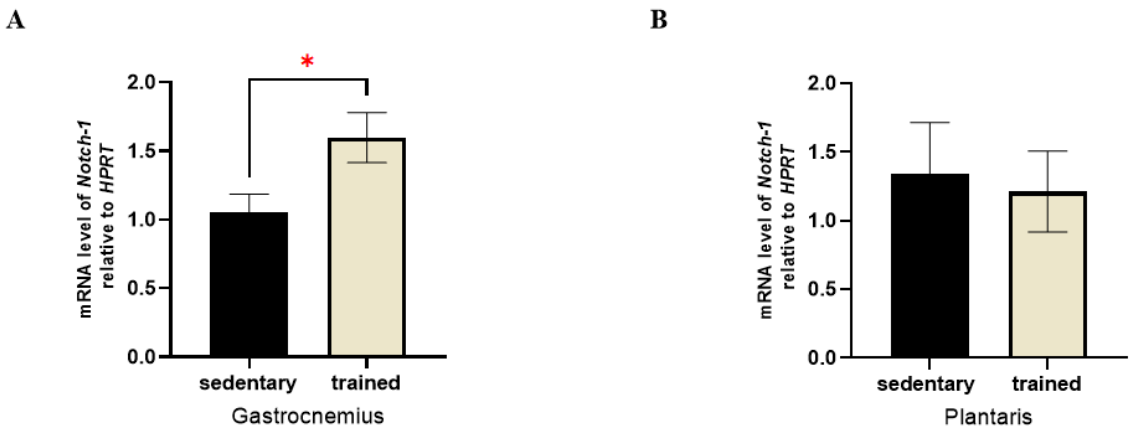


Figure 6.3: Resting levels of mRNA expression of *Notch-1* increases after 9 weeks of training in the gastrocnemius, but not in the plantaris of first set C57BL6 female mice. Effects of 9 weeks of endurance training in first set C57BL6 female mice muscles. Resting levels (72 hours after last bout of exercise) of mRNA expression of *Notch-1* were measured in the gastrocnemius and plantaris. (A) t-tests for quantitative analyses for gastrocnemius and (B) t-tests for quantitative analyses for plantaris. Data shows mean +/- S.E.M., n=6 for plantaris and n=7 for gastrocnemius, * Indicates significative differences P<0.05.

Sedentary INPUT	29.4	30.8	29.7	31.0
Trained INPUT	32.4	27.9	30.7	32.3
H3 INPUT	29.8			

Sedentary ChIP	27.7	28.2	27.1	27.1
Trained ChIP	28.0	26.6	26.0	28.2
H3 ChIP	26.0			

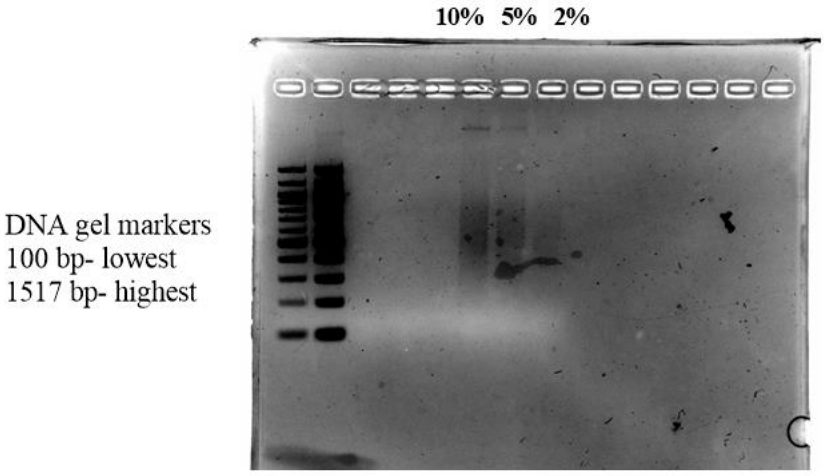
Figure 6.4: Average CT (cycle threshold) values of ChIP products and INPUTs. CT values (cycle threshold) of ChIP and INPUT products measuring H3K27me3 marks and H3 (positive control) on TSS of *Kdr* gene in the gastrocnemius of sedentary and 9 weeks of endurance trained first set C57BL6 female mice. CT values determined by qPCR.

chip product diluted 1:2	ng/ul	*2
sed 3	1.55	3.10
sed 4	3.03	6.06
sed 5	1.36	2.71
sed 7	0.84	1.67
tr 1	0.85	1.70
tr 2	0.98	1.97
tr 3	2.64	5.28
tr 4	0.22	0.43
H3	2.66	5.32

input 20%	ng/µL
sed 3	0.82
sed 4	0.37
sed 5	0.83
sed 7	0.14
tr 1	0.62
tr 2	1.45
tr 3	0.90
tr 4	1.38
H3	0.85

Figure 6.5: DNA quantifications (ng/µl) of ChIP products and 20% INPUTs from chromatin extracts from skeletal muscles. Average DNA concentration values (ng/µl) of ChIP products and 20% input. ChIP products were diluted 1:2 for DNA quantification. First set C57BL6 female mice sedentary and 9 weeks trained gastrocnemius muscles used. Low and variable concentration could be due to insufficient chromatin extraction.

A



B

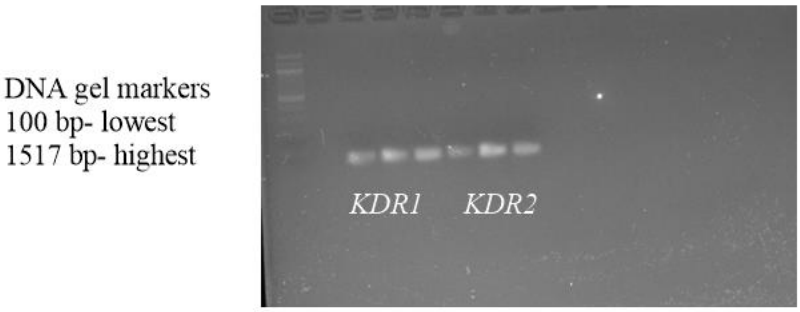


Figure 6.6: DNA gels for chromatin extracts from skeletal muscle and qPCR products for ChIP assessing ChIP fragment sizes. 2% agarose gel loaded (A) chromatin extracts from gastrocnemius of first set C57BL6 female mice in order of: 10% total chromatin extract, 5% total chromatin extract, and 2% total chromatin extracts, extracts range around 400-600 bps. (B) purified PCR products from ChIP-qPCR assessing fragment sizes using KDR1 and KDR 2 probes (ran in triplicates), fragment size under 100 bps.

References

Accili, Domenico, and Karen C. Arden. "FoxOs at the Crossroads of Cellular Metabolism, Differentiation, and Transformation." *Cell* 117, no. 4 (May 14, 2004): 421–26. https://doi.org/10.1016/S0092-8674(04)00452-0.

Adair, Thomas H., and Jean-Pierre Montani. *Overview of Angiogenesis*. Morgan & Claypool Life Sciences, 2010. https://www.ncbi.nlm.nih.gov/books/NBK53238/.

Adams, Volker, Bernhard Reich, Madlen Uhlemann, and Josef Niebauer. "Molecular Effects of Exercise Training in Patients with Cardiovascular Disease: Focus on Skeletal Muscle, Endothelium, and Myocardium." *American Journal of Physiology-Heart and Circulatory Physiology* 313, no. 1 (July 2017): H72–88. https://doi.org/10.1152/ajpheart.00470.2016.

Aiken, Julian, Emilie Roudier, Joseph Ciccone, Genevieve Drouin, Anna Stromberg, Jovana Vojnovic, I. Mark Olfert, et al. "Phosphorylation of Murine Double Minute-2 on Ser166 Is Downstream of VEGF-A in Exercised Skeletal Muscle and Regulates Primary Endothelial Cell Migration and FoxO Gene Expression." *The FASEB Journal* 30, no. 3 (2016): 1120–34. https://doi.org/10.1096/fj.15-276964.

Ameln, Helene, Thomas Gustafsson, Carl Johan Sundberg, Kensaku Okamoto, Eva Jansson, Lorenz Poellinger, and Yuichi Makino. "Physiological Activation of Hypoxia Inducible Factor-1 in Human Skeletal Muscle." *The FASEB Journal* 19, no. 8 (June 2005): 1009–11. https://doi.org/10.1096/fj.04-2304fje.

Andersen, Pep, and Andries J. Kroese. "Capillary Supply in Soleus and Gastrocnemius Muscles of Man." *Pflügers Archiv* 375, no. 3 (August 1, 1978): 245–49. https://doi.org/10.1007/BF00582437.

Andersen, Per, and Jan Henriksson. "Capillary Supply of the Quadriceps Femoris Muscle of Man: Adaptive Response to Exercise." *The Journal of Physiology* 270, no. 3 (September 1, 1977): 677–90. https://doi.org/10.1113/jphysiol.1977.sp011975.

Andrade, Jorge, Chenyue Shi, Ana S. H. Costa, Jeongwoon Choi, Jaeryung Kim, Anuradha Doddaballapur, Toshiya Sugino, et al. "Control of Endothelial Quiescence by FOXO-Regulated Metabolites." *Nature Cell Biology* 23, no. 4 (April 2021): 413–23. https://doi.org/10.1038/s41556-021-00637-6.

Arany, Zoltan, Shi-Yin Foo, Yanhong Ma, Jorge L. Ruas, Archana Bommi-Reddy, Geoffrey Girnun, Marcus Cooper, et al. "HIF-Independent Regulation of VEGF and Angiogenesis by the Transcriptional Coactivator PGC-1α." *Nature* 451, no. 7181 (February 2008): 1008–12. https://doi.org/10.1038/nature06613.

Arena, Giuseppe, Romain Riscal, Laetitia K. Linares, and Laurent Le Cam. "MDM2 Controls Gene Expression Independently of P53 in Both Normal and Cancer Cells." *Cell Death and Differentiation* 25, no. 9 (September 2018): 1533–35. https://doi.org/10.1038/s41418-018-0156-x.

Ballard, Heather J. "ATP and Adenosine in the Regulation of Skeletal Muscle Blood Flow during Exercise." *Sheng Li Xue Bao: [Acta Physiologica Sinica]* 66, no. 1 (February 25, 2014): 67–78.

Bannister, Andrew J, and Tony Kouzarides. "Regulation of Chromatin by Histone Modifications." *Cell Research* 21, no. 3 (March 2011): 381–95. https://doi.org/10.1038/cr.2011.22.

Barnouin, Yoann, Jamie S. McPhee, Gillian Butler-Browne, Alessandra Bosutti, Giuseppe De Vito, David A. Jones, Marco Narici, Anthony Behin, Jean-Yves Hogrel, and Hans Degens. "Coupling between Skeletal Muscle Fiber Size and Capillarization Is Maintained during Healthy Aging." *Journal of Cachexia, Sarcopenia and Muscle* 8, no. 4 (August 2017): 647–59. https://doi.org/10.1002/jcsm.12194.

Barrès, Romain, Megan E. Osler, Jie Yan, Anna Rune, Tomas Fritz, Kenneth Caidahl, Anna Krook, and Juleen R. Zierath. "Non-CpG Methylation of the PGC-1alpha Promoter through DNMT3B Controls Mitochondrial Density." *Cell Metabolism* 10, no. 3 (September 2009): 189–98. https://doi.org/10.1016/j.cmet.2009.07.011.

Barrès, Romain, Jie Yan, Brendan Egan, Jonas Thue Treebak, Morten Rasmussen, Tomas Fritz, Kenneth Caidahl, Anna Krook, Donal J. O'Gorman, and Juleen R. Zierath. "Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle." *Cell Metabolism* 15, no. 3 (March 2012): 405–11. https://doi.org/10.1016/j.cmet.2012.01.001.

Baum, Oliver, Jennifer Gübeli, Sebastian Frese, Eleonora Torchetti, Corinna Malik, Adolfo Odriozola, Franziska Graber, Hans Hoppeler, and Stefan A. Tschanz. "Angiogenesis-Related Ultrastructural Changes to Capillaries in Human Skeletal Muscle in Response to Endurance Exercise." *Journal of Applied Physiology* 119, no. 10 (November 15, 2015): 1118–26. https://doi.org/10.1152/japplphysiol.00594.2015.

Birot, Olivier J. G., Nathalie Koulmann, André Peinnequin, and Xavier A. Bigard. "Exercise-Induced Expression of Vascular Endothelial Growth Factor MRNA in Rat Skeletal Muscle Is Dependent on Fibre Type." *The Journal of Physiology* 552, no. 1 (2003): 213–21. https://doi.org/10.1113/jphysiol.2003.043026.

Breen, E. C., E. C. Johnson, H. Wagner, H. M. Tseng, L. A. Sung, and P. D. Wagner. "Angiogenic Growth Factor MRNA Responses in Muscle to a Single Bout of Exercise." *Journal of Applied Physiology* 81, no. 1 (July 1, 1996): 355–61. https://doi.org/10.1152/jappl.1996.81.1.355.

Brenkman, Arjan B., Peter L. J. de Keizer, Niels J. F. van den Broek, A. G. Jochemsen, and Boudewijn M. Th. Burgering. "Mdm2 Induces Mono-Ubiquitination of FOXO4." *PLoS ONE* 3, no. 7 (July 30, 2008): e2819. https://doi.org/10.1371/journal.pone.0002819.

Brinkman, Arie B., Hongcang Gu, Stefanie J.J. Bartels, Yingying Zhang, Filomena Matarese, Femke Simmer, Hendrik Marks, et al. "Sequential ChIP-Bisulfite Sequencing Enables Direct Genome-Scale Investigation of Chromatin and DNA Methylation Cross-Talk." *Genome Research* 22, no. 6 (June 2012): 1128–38. https://doi.org/10.1101/gr.133728.111.

Brooks, Susan V. "Current Topics for Teaching Skeletal Muscle Physiology." *Advances in Physiology Education* 27, no. 4 (December 2003): 171–82. https://doi.org/10.1152/advan.2003.27.4.171.

Caiafa, Paola, and Michele Zampieri. "DNA Methylation and Chromatin Structure: The Puzzling CpG Islands." *Journal of Cellular Biochemistry* 94, no. 2 (2005): 257–65. https://doi.org/10.1002/jcb.20325.

Cao, Jian, and Qin Yan. "Histone Ubiquitination and Deubiquitination in Transcription, DNA Damage Response, and Cancer." *Frontiers in Oncology* 2 (2012). https://www.frontiersin.org/articles/10.3389/fonc.2012.00026.

Cao, Ye-Chi, Su-Kang Shan, Bei Guo, Chang-Chun Li, Fu-Xing-Zi Li, Ming-Hui Zheng, Qiu-Shuang Xu, et al. "Histone Lysine Methylation Modification and Its Role in Vascular Calcification." *Frontiers in Endocrinology* 13 (June 16, 2022): 863708. https://doi.org/10.3389/fendo.2022.863708.

Caretti, Giuseppina, Monica Di Padova, Bruce Micales, Gary E. Lyons, and Vittorio Sartorelli. "The Polycomb Ezh2 Methyltransferase Regulates Muscle Gene Expression and Skeletal Muscle Differentiation." *Genes & Development* 18, no. 21 (November 1, 2004): 2627–38. https://doi.org/10.1101/gad.1241904.

Cell Signaling Technology. "Overview of Chromatin Immunoprecipitation (ChIP)." Accessed November 9, 2022. https://www.cellsignal.com/applications/chip-and-chip-seq/regulation-expression-in-cell-and-tissue.

Charles, M., N. Charifi, J. Verney, V. Pichot, L. Feasson, F. Costes, and C. Denis. “Effect of Endurance Training on Muscle Microvascular Filtration Capacity and Vascular Bed Morphometry in the Elderly.” *Acta Physiologica* 187, no. 3 (July 2006): 399–406. https://doi.org/10.1111/j.1748-1716.2006.01585.x.

Chen, Delin, Muyang Li, Jianyuan Luo, and Wei Gu. “Direct Interactions between HIF-1α and Mdm2 Modulate P53 Function *.” *Journal of Biological Chemistry* 278, no. 16 (April 18, 2003): 13595–98. https://doi.org/10.1074/jbc.C200694200.

Clifford, Philip S., and Ylva Hellsten. “Vasodilatory Mechanisms in Contracting Skeletal Muscle.” *Journal of Applied Physiology* 97, no. 1 (July 1, 2004): 393–403. https://doi.org/10.1152/japplphysiol.00179.2004.

Cornelissen, V. A., B. Verheyden, A. E. Aubert, and R. H. Fagard. “Effects of Aerobic Training Intensity on Resting, Exercise and Post-Exercise Blood Pressure, Heart Rate and Heart-Rate Variability.” *Journal of Human Hypertension* 24, no. 3 (March 2010): 175–82. https://doi.org/10.1038/jhh.2009.51.

Deaton, Aimée M., and Adrian Bird. “CpG Islands and the Regulation of Transcription.” *Genes & Development* 25, no. 10 (May 15, 2011): 1010–22. https://doi.org/10.1101/gad.2037511.

Dillon, Shane C., Xing Zhang, Raymond C. Trievel, and Xiaodong Cheng. “The SET-Domain Protein Superfamily: Protein Lysine Methyltransferases.” *Genome Biology* 6, no. 8 (August 2, 2005): 227. https://doi.org/10.1186/gb-2005-6-8-227.

Dohmesen, Christoph, Max Koeppel, and Matthias Dobbelstein. “Specific Inhibition of Mdm2-Mediated Neddylation by Tip60.” *Cell Cycle* 7, no. 2 (January 15, 2008): 222–31. https://doi.org/10.4161/cc.7.2.5185.

Duan, Ran, Wenfang Du, and Weijian Guo. “EZH2: A Novel Target for Cancer Treatment.” *Journal of Hematology & Oncology* 13 (July 28, 2020): 104. https://doi.org/10.1186/s13045-020-00937-8.

Duscha, Brian D, William E Kraus, Steven J Keteyian, Martin J Sullivan, Howard J Green, Fred H Schachat, Anne M Pippen, Clinton A Brawner, Jason M Blank, and Brian H Annex. “Capillary Density of Skeletal Muscle.” *Journal of the American College of Cardiology* 33, no. 7 (June 1999): 1956–63. https://doi.org/10.1016/S0735-1097(99)00101-1.

Egginton, S. “Physiological Factors Influencing Capillary Growth: Physiological Angiogenesis.” *Acta Physiologica* 202, no. 3 (July 2011): 225–39. https://doi.org/10.1111/j.1748-1716.2010.02194.x.

Egginton, S., O. Hudlická, M. D. Brown, H. Walter, J. B. Weiss, and A. Bate. “Capillary Growth in Relation to Blood Flow and Performance in Overloaded Rat Skeletal Muscle.” *Journal of Applied Physiology (Bethesda, Md.: 1985)* 85, no. 6 (December 1998): 2025–32. https://doi.org/10.1152/jappl.1998.85.6.2025.

Egginton, Stuart. “Invited Review: Activity-Induced Angiogenesis.” *Pflügers Archiv - European Journal of Physiology* 457, no. 5 (August 13, 2008): 963. https://doi.org/10.1007/s00424-008-0563-9.

Essén, B., E. Jansson, J. Henriksson, A. W. Taylor, and B. Saltin. “Metabolic Characteristics of Fibre Types in Human Skeletal Muscle.” *Acta Physiologica Scandinavica* 95, no. 2 (October 1975): 153–65. https://doi.org/10.1111/j.1748-1716.1975.tb10038.x.

Even-Faitelson, Liron, Vahideh Hassan-Zadeh, Zahra Baghestani, and David P. Bazett-Jones. “Coming to Terms with Chromatin Structure.” *Chromosoma* 125, no. 1 (March 1, 2016): 95–110. https://doi.org/10.1007/s00412-015-0534-9.

Fåhraeus, R., and V. Olivares-Illana. “MDM2’s Social Network.” *Oncogene* 33, no. 35 (August 2014): 4365–76. https://doi.org/10.1038/onc.2013.410.

Farhan, Mohd, Marta Silva, Xing Xingan, Yu Huang, and Wenhua Zheng. "Role of FOXO Transcription Factors in Cancer Metabolism and Angiogenesis." *Cells* 9, no. 7 (June 30, 2020): 1586. https://doi.org/10.3390/cells9071586.

Felmeden, D. "Angiogenesis: Basic Pathophysiology and Implications for Disease." *European Heart Journal* 24, no. 7 (April 2003): 586–603. https://doi.org/10.1016/S0195-668X(02)00635-8.

Ferrara, Napoleone. "Role of Vascular Endothelial Growth Factor in the Regulation of Angiogenesis." *Kidney International* 56, no. 3 (September 1999): 794–814. https://doi.org/10.1046/j.1523-1755.1999.00610.x.

Frontera, Walter R., and Julien Ochala. "Skeletal Muscle: A Brief Review of Structure and Function." *Calcified Tissue International* 96, no. 3 (March 2015): 183–95. https://doi.org/10.1007/s00223-014-9915-y.

Gaughan, L. "Regulation of Androgen Receptor and Histone Deacetylase 1 by Mdm2-Mediated Ubiquitylation." *Nucleic Acids Research* 33, no. 1 (January 7, 2005): 13–26. https://doi.org/10.1093/nar/gki141.

Gavin, T. P., J. L. Drew, C. J. Kubik, W. E. Pofahl, and R. C. Hickner. "Acute Resistance Exercise Increases Skeletal Muscle Angiogenic Growth Factor Expression." *Acta Physiologica* 191, no. 2 (October 2007): 139–46. https://doi.org/10.1111/j.1748-1716.2007.01723.x.

Gavin, Timothy P., Christopher B. Robinson, Robert C. Yeager, Justin A. England, L. Wiley Nifong, and Robert C. Hickner. "Angiogenic Growth Factor Response to Acute Systemic Exercise in Human Skeletal Muscle." *Journal of Applied Physiology* 96, no. 1 (January 2004): 19–24. https://doi.org/10.1152/japplphysiol.00748.2003.

Gavin, Timothy P., Howard W. Stallings, Kevin A. Zwetsloot, Lenna M. Westerkamp, Nicholas A. Ryan, Rebecca A. Moore, Walter E. Pofahl, and Robert C. Hickner. "Lower Capillary Density but No Difference in VEGF Expression in Obese vs. Lean Young Skeletal Muscle in Humans." *Journal of Applied Physiology* 98, no. 1 (January 2005): 315–21. https://doi.org/10.1152/japplphysiol.00353.2004.

Gavin, Timothy P., and Peter D. Wagner. "Effect of Short-Term Exercise Training on Angiogenic Growth Factor Gene Responses in Rats." *Journal of Applied Physiology* 90, no. 4 (April 1, 2001): 1219–26. https://doi.org/10.1152/jappl.2001.90.4.1219.

Gorski, Tatiane, and Katrien De Bock. "Metabolic Regulation of Exercise-Induced Angiogenesis." *Vascular Biology* 1, no. 1 (April 1, 2019): H1–8. https://doi.org/10.1530/VB-19-0008.

Gulri, M. (2020). *Investigating the role of selected epigenetic silencing marks in determining mouse skeletal muscle oxidative phenotype* (Masters Thesis, York University). http://hdl.handle.net/10315/37891.

Griffiths, Samuel C., Rebekka A. Schwab, Kamel El Omari, Benjamin Bishop, Ellen J. Iverson, Tomas Malinauskas, Ramin Dubey, et al. "Hedgehog-Interacting Protein Is a Multimodal Antagonist of Hedgehog Signalling." *Nature Communications* 12, no. 1 (December 9, 2021): 7171. https://doi.org/10.1038/s41467-021-27475-2.

Haas, Tara L., and Emmanuel Nwadozi. "Regulation of Skeletal Muscle Capillary Growth in Exercise and Disease." *Applied Physiology, Nutrition, and Metabolism* 40, no. 12 (December 1, 2015): 1221–32. https://doi.org/10.1139/apnm-2015-0336.

Hansen-Smith, Fay M., Olga Hudlicka, and Stuart Egginton. "In Vivo Angiogenesis in Adult Rat Skeletal Muscle: Early Changes in Capillary Network Architecture and Ultrastructure." *Cell and Tissue Research* 286, no. 1 (September 1, 1996): 123–36. https://doi.org/10.1007/s004410050681.

Hellsten, Ylva, and Michael Nyberg. “Cardiovascular Adaptations to Exercise Training.” In *Comprehensive Physiology*, 1–32. John Wiley & Sons, Ltd, 2015. https://doi.org/10.1002/cphy.c140080.

Hendrickse, Paul, and Hans Degens. “The Role of the Microcirculation in Muscle Function and Plasticity.” *Journal of Muscle Research and Cell Motility* 40, no. 2 (2019): 127–40. https://doi.org/10.1007/s10974-019-09520-2.

Hepple, R. T., S. L. M. Mackinnon, J. M. Goodman, S. G. Thomas, and M. J. Plyley. “Resistance and Aerobic Training in Older Men: Effects OnV˙o 2 Peak and the Capillary Supply to Skeletal Muscle.” *Journal of Applied Physiology* 82, no. 4 (April 1997): 1305–10. https://doi.org/10.1152/jappl.1997.82.4.1305.

Hepple, R. T., S. L. M. Mackinnon, S. G. Thomas, J. M. Goodman, and M. J. Plyley. “Quantitating the Capillary Supply and the Response to Resistance Training in Older Men.” *Pflügers Archiv* 433, no. 3 (December 1, 1996): 238–44. https://doi.org/10.1007/s004240050273.

Hershko, Avram, and Aaron Ciechanover. “THE UBIQUITIN SYSTEM.” *Annual Review of Biochemistry* 67, no. 1 (June 1998): 425–79. https://doi.org/10.1146/annurev.biochem.67.1.425.

Hoier, B, N Nordsborg, S Andersen, L Jensen, L Nybo, J Bangsbo, and Y Hellsten. “Pro- and Anti-Angiogenic Factors in Human Skeletal Muscle in Response to Acute Exercise and Training.” *The Journal of Physiology* 590, no. Pt 3 (February 1, 2012): 595–606. https://doi.org/10.1113/jphysiol.2011.216135.

Høier, Birgitte, Karina Olsen, Michael Nyberg, Jens Bangsbo, and Ylva Hellsten. “Contraction-Induced Secretion of VEGF from Skeletal Muscle Cells Is Mediated by Adenosine.” *American Journal of Physiology-Heart and Circulatory Physiology* 299, no. 3 (September 2010): H857–62. https://doi.org/10.1152/ajpheart.00082.2010.

Holloszy, J. O. “Biochemical Adaptations in Muscle. Effects of Exercise on Mitochondrial Oxygen Uptake and Respiratory Enzyme Activity in Skeletal Muscle.” *The Journal of Biological Chemistry* 242, no. 9 (May 10, 1967): 2278–82.

Hosaka, Taisuke, William H. Biggs, David Tieu, Antonia D. Boyer, Nissi M. Varki, Webster K. Cavenee, and Karen C. Arden. “Disruption of Forkhead Transcription Factor (FOXO) Family Members in Mice Reveals Their Functional Diversification.” *Proceedings of the National Academy of Sciences of the United States of America* 101, no. 9 (March 2, 2004): 2975–80. https://doi.org/10.1073/pnas.0400093101.

Huang, Chang, and Bing Zhu. “H3.3 Turnover: A Mechanism to Poise Chromatin for Transcription, or a Response to Open Chromatin?: Prospects & Overviews.” *BioEssays* 36, no. 6 (June 2014): 579–84. https://doi.org/10.1002/bies.201400005.

Hudlicka, Olga. “Microcirculation in Skeletal Muscle.” *Muscles, Ligaments and Tendons Journal* 1, no. 1 (October 30, 2011): 3–11.

Hudlicka, O., Brown, M., & Egginton, S. (1992). Angiogenesis in skeletal and cardiac muscle. *Physiological Reviews*, *72*(2), 369–417. https://doi.org/10.1152/physrev.1992.72.2.369.

Jeltsch, Albert, Julian Broche, and Pavel Bashtrykov. “Molecular Processes Connecting DNA Methylation Patterns with DNA Methyltransferases and Histone Modifications in Mammalian Genomes.” *Genes* 9, no. 11 (November 2018): 566. https://doi.org/10.3390/genes9110566.

Ji, James W., Nikolaos M. Tsoukias, Daniel Goldman, and Aleksander S. Popel. “A Computational Model of Oxygen Transport in Skeletal Muscle for Sprouting and Splitting Modes of Angiogenesis.” *Journal of Theoretical Biology* 241, no. 1 (July 2006): 94–108. https://doi.org/10.1016/j.jtbi.2005.11.019.

Juan, Aster H., Assia Derfoul, Xuesong Feng, James G. Ryall, Stefania Dell'Orso, Alessandra Pasut, Hossein Zare, James M. Simone, Michael A. Rudnicki, and Vittorio Sartorelli. "Polycomb EZH2 Controls Self-Renewal and Safeguards the Transcriptional Identity of Skeletal Muscle Stem Cells." *Genes & Development* 25, no. 8 (April 15, 2011): 789–94. https://doi.org/10.1101/gad.2027911.

Juan, Aster H., Roshan M. Kumar, Joseph G. Marx, Richard A. Young, and Vittorio Sartorelli. "Mir-214-Dependent Regulation of the Polycomb Protein Ezh2 in Skeletal Muscle and Embryonic Stem Cells." *Molecular Cell* 36, no. 1 (October 9, 2009): 61–74. https://doi.org/10.1016/j.molcel.2009.08.008.

Kanki, Yasuharu, Masashi Muramatsu, Yuri Miyamura, Kenta Kikuchi, Yoshiki Higashijima, Ryo Nakaki, Jun-ichi Suehiro, et al. "Bivalent-Histone-Marked Immediate-Early Gene Regulation Is Vital for VEGF-Responsive Angiogenesis." *Cell Reports* 38, no. 6 (February 2022): 110332. https://doi.org/10.1016/j.celrep.2022.110332.

Kanzleiter, Timo, Markus Jähnert, Gunnar Schulze, Joachim Selbig, Nicole Hallahan, Robert Wolfgang Schwenk, and Annette Schürmann. "Exercise Training Alters DNA Methylation Patterns in Genes Related to Muscle Growth and Differentiation in Mice." *American Journal of Physiology-Endocrinology and Metabolism* 308, no. 10 (May 15, 2015): E912–20. https://doi.org/10.1152/ajpendo.00289.2014.

Kawano, Fuminori. "Histone Modification: A Mechanism for Regulating Skeletal Muscle Characteristics and Adaptive Changes." *Applied Sciences* 11, no. 9 (January 2021): 3905. https://doi.org/10.3390/app11093905.

Kawano, Fuminori, Keisuke Nimura, Saki Ishino, Naoya Nakai, Ken Nakata, and Yoshinobu Ohira. "Differences in Histone Modifications between Slow- and Fast-Twitch Muscle of Adult Rats and Following Overload, Denervation, or Valproic Acid Administration." *Journal of Applied Physiology* 119, no. 10 (November 15, 2015): 1042–52. https://doi.org/10.1152/japplphysiol.00289.2015.

Kumar, Hemant, and Dong-Kug Choi. "Hypoxia Inducible Factor Pathway and Physiological Adaptation: A Cell Survival Pathway?" *Mediators of Inflammation* 2015 (2015): 1–11. https://doi.org/10.1155/2015/584758.

Kume, Tsutomu. "Novel Insights into the Differential Functions of Notch Ligands in Vascular Formation." *Journal of Angiogenesis Research* 1, no. 1 (2009): 8. https://doi.org/10.1186/2040-2384-1-8.

Kwon, Duk-Hwa, Gwang Hyeon Eom, Jeong Hyeon Ko, Sera Shin, Hosouk Joung, Nakwon Choe, Yoon Seok Nam, et al. "MDM2 E3 Ligase-Mediated Ubiquitination and Degradation of HDAC1 in Vascular Calcification." *Nature Communications* 7, no. 1 (February 1, 2016): 10492. https://doi.org/10.1038/ncomms10492.

Lam, Brian, Emmanuel Nwadozi, Tara L. Haas, Olivier Birot, and Emilie Roudier. "High Glucose Treatment Limits Drosha Protein Expression and Alters AngiomiR Maturation in Microvascular Primary Endothelial Cells via an Mdm2-Dependent Mechanism." *Cells* 10, no. 4 (March 27, 2021): 742. https://doi.org/10.3390/cells10040742.

Latroche, Claire, Cyril Gitiaux, Fabrice Chrétien, Isabelle Desguerre, Rémi Mounier, and Bénédicte Chazaud. "Skeletal Muscle Microvasculature: A Highly Dynamic Lifeline." *Physiology* 30, no. 6 (November 2015): 417–27. https://doi.org/10.1152/physiol.00026.2015.

Lavie, Carl J., Ross Arena, Damon L. Swift, Neil M. Johannsen, Xuemei Sui, Duck-chul Lee, Conrad P. Earnest, et al. "Exercise and the Cardiovascular System: Clinical Science and Cardiovascular Outcomes." *Circulation Research* 117, no. 2 (July 3, 2015): 207–19. https://doi.org/10.1161/CIRCRESAHA.117.305205.

Leick, Lotte, Ylva Hellsten, Joachim Fentz, Stine S. Lyngby, Jørgen F. P. Wojtaszewski, Juan Hidalgo, and Henriette Pilegaard. "PGC-1α Mediates Exercise-Induced Skeletal Muscle VEGF Expression in Mice." *American Journal of Physiology-Endocrinology and Metabolism* 297, no. 1 (July 2009): E92–103. https://doi.org/10.1152/ajpendo.00076.2009.

Lemieux, Pierre, and Olivier Birot. "Altitude, Exercise, and Skeletal Muscle Angio-Adaptive Responses to Hypoxia: A Complex Story." *Frontiers in Physiology* 12 (2021): 1461. https://doi.org/10.3389/fphys.2021.735557.

Lemieux, Pierre, Emilie Roudier, and Olivier Birot. "Angiostatic Freeze or Angiogenic Move? Acute Cold Stress Prevents Angiokine Secretion from Murine Myotubes but Primes Primary Endothelial Cells for Greater Migratory Capacity." *Frontiers in Physiology* 13 (October 17, 2022): 975652. https://doi.org/10.3389/fphys.2022.975652.

Liang, Huiyun, and Walter F. Ward. "PGC-1α: A Key Regulator of Energy Metabolism." *Advances in Physiology Education* 30, no. 4 (December 2006): 145–51. https://doi.org/10.1152/advan.00052.2006.

Lim, Changhyun, Junya Shimizu, Fuminori Kawano, Hyo Jeong Kim, and Chang Keun Kim. "Adaptive Responses of Histone Modifications to Resistance Exercise in Human Skeletal Muscle." *PLOS ONE* 15, no. 4 (April 9, 2020): e0231321. https://doi.org/10.1371/journal.pone.0231321.

Lindholm, M. E., H. Fischer, L. Poellinger, R. S. Johnson, T. Gustafsson, C. J. Sundberg, and H. Rundqvist. "Negative Regulation of HIF in Skeletal Muscle of Elite Endurance Athletes: A Tentative Mechanism Promoting Oxidative Metabolism." *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 307, no. 3 (August 1, 2014): R248–55. https://doi.org/10.1152/ajpregu.00036.2013.

Lindholm, Malene E., and Helene Rundqvist. "Skeletal Muscle Hypoxia-Inducible Factor-1 and Exercise." *Experimental Physiology* 101, no. 1 (2016): 28–32. https://doi.org/10.1113/EP085318.

Liu, P., Shuaib, M., Zhang, H., Nadeef, S., & Orlando, V. (2019). Ubiquitin ligases HUWE1 and NEDD4 cooperatively control signal-dependent PRC2-Ezh1α/β-mediated adaptive stress response pathway in skeletal muscle cells. *Epigenetics & Chromatin*, *12*, 78. https://doi.org/10.1186/s13072-019-0322-5.

Maasar, Mohd-Firdaus, Daniel C. Turner, Piotr P. Gorski, Robert A. Seaborne, Juliette A. Strauss, Sam O. Shepherd, Matt Cocks, et al. "The Comparative Methylome and Transcriptome After Change of Direction Compared to Straight Line Running Exercise in Human Skeletal Muscle." *Frontiers in Physiology* 12 (February 19, 2021): 619447. https://doi.org/10.3389/fphys.2021.619447.

Malek, Moh H., and I. Mark Olfert. "Global Deletion of Thrombospondin-1 Increases Cardiac and Skeletal Muscle Capillarity and Exercise Capacity in Mice: Muscle Phenotype of TSP-1 Null Mice." *Experimental Physiology* 94, no. 6 (June 1, 2009): 749–60. https://doi.org/10.1113/expphysiol.2008.045989.

Mason, Steven D., Helene Rundqvist, Ioanna Papandreou, Roger Duh, Wayne J. McNulty, Richard A. Howlett, I. Mark Olfert, et al. "HIF-1α in Endurance Training: Suppression of Oxidative Metabolism." *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 293, no. 5 (November 2007): R2059–69. https://doi.org/10.1152/ajpregu.00335.2007.

Masuzawa, Ryo, Ryotaro Konno, Ikumi Ohsawa, Atsuya Watanabe, and Fuminori Kawano. "Muscle Type-Specific RNA Polymerase II Recruitment during PGC-1α Gene Transcription after Acute Exercise in Adult Rats." *Journal of Applied Physiology* 125, no. 4 (October 1, 2018): 1238–45. https://doi.org/10.1152/japplphysiol.00202.2018.

Mathai, Anila S., Arend Bonen, Carley R. Benton, D. L. Robinson, and Terry E. Graham. "Rapid Exercise-Induced Changes in PGC-1α MRNA and Protein in Human Skeletal Muscle." *Journal of Applied Physiology* 105, no. 4 (October 2008): 1098–1105. https://doi.org/10.1152/japplphysiol.00847.2007.

Mazibuko-Mbeje, Sithandiwe Eunice, Phiwayinkosi V. Dludla, Bongani B. Nkambule, Nnini Obonye, and Johan Louw. "The Role of Glucose and Fatty Acid Metabolism in the Development of Insulin Resistance in Skeletal Muscle." In *Muscle Cell and Tissue - Current Status of Research Field*, edited by Kunihiro Sakuma. InTech, 2018. https://doi.org/10.5772/intechopen.75904.

McGee, Sean L, Erin Fairlie, Andrew P Garnham, and Mark Hargreaves. "Exercise-Induced Histone Modifications in Human Skeletal Muscle." *The Journal of Physiology* 587, no. Pt 24 (December 15, 2009): 5951–58. https://doi.org/10.1113/jphysiol.2009.181065.

McGee, Sean L., and Mark Hargreaves. "Epigenetics and Exercise." *Trends in Endocrinology & Metabolism* 30, no. 9 (September 1, 2019): 636–45. https://doi.org/10.1016/j.tem.2019.06.002.

McGee, Sean L., and Ken R. Walder. "Exercise and the Skeletal Muscle Epigenome." *Cold Spring Harbor Perspectives in Medicine* 7, no. 9 (September 2017): a029876. https://doi.org/10.1101/cshperspect.a029876.

Milkiewicz, Malgorzata, Jennifer L Doyle, Tomasz Fudalewski, Eric Ispanovic, Maliheh Aghasi, and Tara L Haas. "HIF-1α and HIF-2α Play a Central Role in Stretch-Induced but Not Shear-Stress-Induced Angiogenesis in Rat Skeletal Muscle." *The Journal of Physiology* 583, no. Pt 2 (September 1, 2007): 753–66. https://doi.org/10.1113/jphysiol.2007.136325.

Milkiewicz, Malgorzata, Olga Hudlicka, Julie Verhaeg, Stuart Egginton, and Margaret D. Brown. "Differential Expression of Flk-1 and Flt-1 in Rat Skeletal Muscle in Response to Chronic Ischaemia: Favourable Effect of Muscle Activity." *Clinical Science (London, England: 1979)* 105, no. 4 (October 2003): 473–82. https://doi.org/10.1042/CS20030035.

Milkiewicz, Malgorzata, Emilie Roudier, Jennifer L. Doyle, Anastassia Trifonova, Olivier Birot, and Tara L. Haas. "Identification of a Mechanism Underlying Regulation of the Anti-Angiogenic Forkhead Transcription Factor FoxO1 in Cultured Endothelial Cells and Ischemic Muscle." *The American Journal of Pathology* 178, no. 2 (February 2011): 935–44. https://doi.org/10.1016/j.ajpath.2010.10.042.

Minsky, Neri, and Moshe Oren. "The RING Domain of Mdm2 Mediates Histone Ubiquitylation and Transcriptional Repression." *Molecular Cell* 16, no. 4 (November 2004): 631–39. https://doi.org/10.1016/j.molcel.2004.10.016.

Moll, Ute M., and Oleksi Petrenko. "The MDM2-P53 Interaction." *Molecular Cancer Research* 1, no. 14 (December 1, 2003): 1001–8.

Morey, Lluís, and Kristian Helin. "Polycomb Group Protein-Mediated Repression of Transcription." *Trends in Biochemical Sciences* 35, no. 6 (June 1, 2010): 323–32. https://doi.org/10.1016/j.tibs.2010.02.009.

Mukund, Kavitha, and Shankar Subramaniam. "Skeletal Muscle: A Review of Molecular Structure and Function, in Health and Disease." *Wiley Interdisciplinary Reviews. Systems Biology and Medicine* 12, no. 1 (2020): e1462. https://doi.org/10.1002/wsbm.1462.

Murrant, Coral L., Nicole M. Fletcher, Eamon J. H. Fitzpatrick, and Kinley S. Gee. "Do Skeletal Muscle Motor Units and Microvascular Units Align to Help Match Blood Flow to Metabolic Demand?" *European Journal of Applied Physiology* 121, no. 5 (May 1, 2021): 1241–54. https://doi.org/10.1007/s00421-021-04598-4.

Myrhage, Rhagnar, and Olga Hudlická. “Capillary Growth in Chronically Stimulated Adult Skeletal Muscle as Studied by Intravital Microscopy and Histological Methods in Rabbits and Rats.” *Microvascular Research* 16, no. 1 (July 1, 1978): 73–90. https://doi.org/10.1016/0026-2862(78)90046-8.

Nakamura, Keisuke, Ikumi Ohsawa, Ryo Masuzawa, Ryotaro Konno, Atsuya Watanabe, and Fuminori Kawano. “Running Training Experience Attenuates Disuse Atrophy in Fast-Twitch Skeletal Muscles of Rats.” *Journal of Applied Physiology* 123, no. 4 (October 1, 2017): 902–13. https://doi.org/10.1152/japplphysiol.00289.2017.

Nieves, Bonnie J., Patricia A. D’Amore, and Brad A. Bryan. “The Function of Vascular Endothelial Growth Factor.” *BioFactors* 35, no. 4 (July 2009): 332–37. https://doi.org/10.1002/biof.46.

O’Carroll, Dónal, Sylvia Erhardt, Michaela Pagani, Sheila C. Barton, M. Azim Surani, and Thomas Jenuwein. “The Polycomb-Group Gene Ezh2 Is Required for Early Mouse Development.” *Molecular and Cellular Biology* 21, no. 13 (July 2001): 4330–36. https://doi.org/10.1128/MCB.21.13.4330-4336.2001.

Ohsawa, Ikumi, and Fuminori Kawano. “Chronic Exercise Training Activates Histone Turnover in Mouse Skeletal Muscle Fibers.” *The FASEB Journal* 35, no. 4 (April 2021). https://doi.org/10.1096/fj.202002027RR.

Ohsawa, Ikumi, Ryotaro Konno, Ryo Masuzawa, and Fuminori Kawano. “Amount of Daily Exercise Is an Essential Stimulation to Alter the Epigenome of Skeletal Muscle in Rats.” *Journal of Applied Physiology* 125, no. 4 (October 1, 2018): 1097–1104. https://doi.org/10.1152/japplphysiol.00074.2018.

Okazaki, K, and H Holtzer. “Myogenesis: Fusion, Myosin Synthesis, and the Mitotic Cycle.” *Proceedings of the National Academy of Sciences* 56, no. 5 (November 1966): 1484–90. https://doi.org/10.1073/pnas.56.5.1484.

Olenich, Sara A., Navarre Gutierrez-Reed, Gerald N. Audet, and I. Mark Olfert. “Temporal Response of Positive and Negative Regulators in Response to Acute and Chronic Exercise Training in Mice.” *The Journal of Physiology* 591, no. 20 (2013): 5157–69. https://doi.org/10.1113/jphysiol.2013.254979.

Olfert, I. Mark, and Olivier Birot. “Importance of Anti-Angiogenic Factors in the Regulation of Skeletal Muscle Angiogenesis: Physical Activity and Inhibitors of Angiogenesis.” *Microcirculation* 18, no. 4 (May 2011): 316–30. https://doi.org/10.1111/j.1549-8719.2011.00092.x.

Olfert, I. Mark, Ellen C. Breen, Timothy P. Gavin, and Peter D. Wagner. “Temporal Thrombospondin-1 MRNA Response in Skeletal Muscle Exposed to Acute and Chronic Exercise.” *Growth Factors* 24, no. 4 (January 1, 2006): 253–59. https://doi.org/10.1080/08977190601000111.

Olfert, I. Mark, Ellen C. Breen, Odile Mathieu-Costello, and Peter D. Wagner. “Skeletal Muscle Capillarity and Angiogenic MRNA Levels after Exercise Training in Normoxia and Chronic Hypoxia.” *Journal of Applied Physiology* 91, no. 3 (September 2001): 1176–84. https://doi.org/10.1152/jappl.2001.91.3.1176.

Olfert, I. Mark, Richard A. Howlett, Kechun Tang, Nancy D. Dalton, Yusu Gu, Kirk L. Peterson, Peter D. Wagner, and Ellen C. Breen. “Muscle-Specific VEGF Deficiency Greatly Reduces Exercise Endurance in Mice: VEGF-Deficiency Reduces Exercise Capacity.” *The Journal of Physiology* 587, no. 8 (April 15, 2009): 1755–67. https://doi.org/10.1113/jphysiol.2008.164384.

Parise, Gianni, Coral L. Murrant, Matthew Cocks, Tim Snijders, Oliver Baum, and Michael J. Plyley. “Capillary Facilitation of Skeletal Muscle Function in Health and Disease.” *Applied Physiology, Nutrition, and Metabolism* 45, no. 5 (May 2020): 453–62. https://doi.org/10.1139/apnm-2019-0416.

Peixoto, Paul, Pierre-François Cartron, Aurélien A. Serandour, and Eric Hervouet. “From 1957 to Nowadays: A Brief History of Epigenetics.” *International Journal of Molecular Sciences* 21, no. 20 (October 14, 2020): 7571. https://doi.org/10.3390/ijms21207571.

Pfaff, Miles J., Subhradip Mukhopadhyay, Mark Hoofnagle, Christine Chabasse, and Rajabrata Sarkar. “Tumor Suppressor Protein P53 Negatively Regulates Ischemia-Induced Angiogenesis and Arteriogenesis.” *Journal of Vascular Surgery* 68, no. 6 (December 2018): 222S-233S.e1. https://doi.org/10.1016/j.jvs.2018.02.055.

Popov, Daniil V., Pavel A. Makhnovskii, Elena I. Shagimardanova, Guzel R. Gazizova, Evgeny A. Lysenko, Oleg A. Gusev, and Olga L. Vinogradova. “Contractile Activity-Specific Transcriptome Response to Acute Endurance Exercise and Training in Human Skeletal Muscle.” *American Journal of Physiology-Endocrinology and Metabolism* 316, no. 4 (April 1, 2019): E605–14. https://doi.org/10.1152/ajpendo.00449.2018.

Potente, Michael, Carmen Urbich, Ken-ichiro Sasaki, Wolf K. Hofmann, Christopher Heeschen, Alexandra Aicher, Ramya Kollipara, Ronald A. DePinho, Andreas M. Zeiher, and Stefanie Dimmeler. “Involvement of Foxo Transcription Factors in Angiogenesis and Postnatal Neovascularization.” *Journal of Clinical Investigation* 115, no. 9 (September 1, 2005): 2382–92. https://doi.org/10.1172/JCI23126.

Riscal, Romain, Emilie Schrepfer, Giuseppe Arena, Madi Y. Cissé, Floriant Bellvert, Maud Heuillet, Florian Rambow, et al. “Chromatin-Bound MDM2 Regulates Serine Metabolism and Redox Homeostasis Independently of P53.” *Molecular Cell* 62, no. 6 (June 16, 2016): 890–902. https://doi.org/10.1016/j.molcel.2016.04.033.

Rothbart, Scott B., and Brian D. Strahl. “Interpreting Thelanguage of Histone and DNA Modifications.” *Biochimica et Biophysica Acta* 1839, no. 8 (August 2014): 627–43. https://doi.org/10.1016/j.bbagrm.2014.03.001.

Roudier, Emilie, Julian Aiken, Dara Slopack, Fares Gouzi, Jacques Mercier, Tara L Haas, Thomas Gustafsson, Maurice Hayot, and Olivier Birot. “Novel Perspective: Exercise Training Stimulus Triggers the Expression of the Oncoprotein Human Double Minute-2 in Human Skeletal Muscle.” *Physiological Reports* 1, no. 2 (July 2013): e00028. https://doi.org/10.1002/phy2.28.

Roudier, Emilie, Paul Forn, Mary Ellen Perry, and Olivier Birot. “Murine Double Minute-2 Expression Is Required for Capillary Maintenance and Exercise-Induced Angiogenesis in Skeletal Muscle.” *The FASEB Journal* 26, no. 11 (November 2012): 4530–39. https://doi.org/10.1096/fj.12-212720.

Roudier, Emilie, Charlotte Gineste, Alexandra Wazna, Kooroush Dehghan, Dominique Desplanches, and Olivier Birot. “Angio-Adaptation in Unloaded Skeletal Muscle: New Insights into an Early and Muscle Type-Specific Dynamic Process.” *The Journal of Physiology* 588, no. Pt 22 (November 15, 2010): 4579–91. https://doi.org/10.1113/jphysiol.2010.193243.

Roudier, Emilie, Malgorzata Milkiewicz, Olivier Birot, Dara Slopack, Andreas Montelius, Thomas Gustafsson, Ji Hye Paik, et al. “Endothelial FoxO1 Is an Intrinsic Regulator of Thrombospondin 1 Expression That Restrains Angiogenesis in Ischemic Muscle.” *Angiogenesis* 16, no. 4 (October 2013): 759–72. https://doi.org/10.1007/s10456-013-9353-x.

Saltin, Bengt, Jan Henriksson, Else Nygaard, Per Andersen, and Eva Jansson. “FIBER TYPES AND METABOLIC POTENTIALS OF SKELETAL MUSCLES IN SEDENTARY MAN AND ENDURANCE RUNNERS.” *Annals of the New York Academy of Sciences* 301, no. 1 The Marathon (October 1977): 3–29. https://doi.org/10.1111/j.1749-6632.1977.tb38182.x.

Schiaffino, Stefano, and Carlo Reggiani. “Fiber Types in Mammalian Skeletal Muscles.” *Physiological Reviews* 91, no. 4 (October 2011): 1447–1531. https://doi.org/10.1152/physrev.00031.2010.

Scott, Wayne, Jennifer Stevens, and Stuart A Binder–Macleod. “Human Skeletal Muscle Fiber Type Classifications.” *Physical Therapy* 81, no. 11 (November 1, 2001): 1810–16. https://doi.org/10.1093/ptj/81.11.1810.

Seals, Douglas R. “Edward F. Adolph Distinguished Lecture: The Remarkable Anti-Aging Effects of Aerobic Exercise on Systemic Arteries.” *Journal of Applied Physiology* 117, no. 5 (September 1, 2014): 425–39. https://doi.org/10.1152/japplphysiol.00362.2014.

Segal, Steven S. “Regulation of Blood Flow in the Microcirculation.” *Microcirculation* 12, no. 1 (January 2, 2005): 33–45. https://doi.org/10.1080/10739680590895028.

Semenza, Gregg L. “Hypoxia-Inducible Factor 1 and Cardiovascular Disease.” *Annual Review of Physiology* 76, no. 1 (February 10, 2014): 39–56. https://doi.org/10.1146/annurev-physiol-021113-170322.

Shi, Lei, Xiangyu Shen, and Yuan Shen. “USP49-Mediated Histone H2B Deubiquitination Regulates HCT116 Cell Proliferation through MDM2-P53 Axis.” *Molecular and Cellular Biology* 42, no. 3 (March 17, 2022): e00434-21. https://doi.org/10.1128/mcb.00434-21.

Shimizu, Junya, and Fuminori Kawano. “Exercise-induced Histone H3 Trimethylation at Lysine 27 Facilitates the Adaptation of Skeletal Muscle to Exercise in Mice.” *The Journal of Physiology* 600, no. 14 (July 2022): 3331–53. https://doi.org/10.1113/JP282917.

Sidossis, Labros S., Robert R. Wolfe, and Andrew R. Coggan. “Regulation of Fatty Acid Oxidation in Untrained vs. Trained Men during Exercise.” *American Journal of Physiology-Endocrinology and Metabolism* 274, no. 3 (March 1998): E510–15. https://doi.org/10.1152/ajpendo.1998.274.3.E510.

Siekmann, Arndt F, and Nathan D Lawson. “Notch Signalling and the Regulation of Angiogenesis.” *Cell Adhesion & Migration* 1, no. 2 (2007): 104–6.

Sjøgaard, G. “Capillary Supply and Cross-Sectional Area of Slow and Fast Twitch Muscle Fibres in Man.” *Histochemistry* 76, no. 4 (December 1, 1982): 547–55. https://doi.org/10.1007/BF00489909.

Slopack, Dara, Emilie Roudier, Sammy T. K. Liu, Emmanuel Nwadozi, Olivier Birot, and Tara L. Haas. “Forkhead BoxO Transcription Factors Restrain Exercise-Induced Angiogenesis: FoxO Proteins Delay Exercise-Driven Angiogenesis.” *The Journal of Physiology* 592, no. 18 (September 15, 2014): 4069–82. https://doi.org/10.1113/jphysiol.2014.275867.

Small, Lewin, Lars R. Ingerslev, Eleonora Manitta, Rhianna C. Laker, Ann N. Hansen, Brendan Deeney, Alain Carrié, Philippe Couvert, and Romain Barrès. “Ablation of DNA-Methyltransferase 3A in Skeletal Muscle Does Not Affect Energy Metabolism or Exercise Capacity.” Edited by Yvonne Böttcher. *PLOS Genetics* 17, no. 1 (January 29, 2021): e1009325. https://doi.org/10.1371/journal.pgen.1009325.

Snyder, Nathan A., and Gustavo M. Silva. “Deubiquitinating Enzymes (DUBs): Regulation, Homeostasis, and Oxidative Stress Response.” *Journal of Biological Chemistry* 297, no. 3 (September 1, 2021): 101077. https://doi.org/10.1016/j.jbc.2021.101077.

Solomon, Elizabeth R, Kevin K Caldwell, and Andrea M Allan. “A Novel Method for the Normalization of ChIP-QPCR Data.” *MethodsX* 8 (2021): 101504. https://doi.org/10.1016/j.mex.2021.101504.

Stojic, Lovorka, Zuzana Jasencakova, Carolina Prezioso, Alexandra Stützer, Beatrice Bodega, Diego Pasini, Rebecca Klingberg, et al. “Chromatin Regulated Interchange between Polycomb Repressive Complex 2

(PRC2)-Ezh2 and PRC2-Ezh1 Complexes Controls Myogenin Activation in Skeletal Muscle Cells." *Epigenetics & Chromatin* 4 (September 5, 2011): 16. https://doi.org/10.1186/1756-8935-4-16.

Straface, Giuseppe, Tamar Aprahamian, Andrea Flex, Eleonora Gaetani, Federico Biscetti, Roy C. Smith, Giovanni Pecorini, et al. "Sonic Hedgehog Regulates Angiogenesis and Myogenesis during Post-natal Skeletal Muscle Regeneration." *Journal of Cellular and Molecular Medicine* 13, no. 8b (August 2009): 2424–35. https://doi.org/10.1111/j.1582-4934.2008.00440.x.

Stroka, Deborah M., Tobias Burkhardt, Isabelle Desbaillets, Roland H. Wenger, Desley A. H. Neil, Christian Bauer, Max Gassmann, and Daniel Candinas. "HIF-1 Is Expressed in Normoxic Tissue and Displays an Organ-specific Regulation under Systemic Hypoxia." *The FASEB Journal* 15, no. 13 (November 2001): 2445–53. https://doi.org/10.1096/fj.01-0125com.

Turner, D. C., P. P. Gorski, M. F. Maasar, R. A. Seaborne, P. Baumert, A. D. Brown, M. O. Kitchen, et al. "DNA Methylation across the Genome in Aged Human Skeletal Muscle Tissue and Muscle-Derived Cells: The Role of HOX Genes and Physical Activity." *Scientific Reports* 10, no. 1 (September 21, 2020): 15360. https://doi.org/10.1038/s41598-020-72730-z.

Uckelmann, Michael, and Chen Davidovich. "Not Just a Writer: PRC2 as a Chromatin Reader." *Biochemical Society Transactions* 49, no. 3 (June 30, 2021): 1159–70. https://doi.org/10.1042/BST20200728.

Vaikevicius. "Effects of Age and Aerobic Capacity on Arterial Stiffness in Healthy Adults.," 1993. https://doi.org/10.1161/01.CIR.88.4.1456.

Wagenmakers, Anton J. M., Juliette A. Strauss, Sam O. Shepherd, Michelle A. Keske, and Matthew Cocks. "Increased Muscle Blood Supply and Transendothelial Nutrient and Insulin Transport Induced by Food Intake and Exercise: Effect of Obesity and Ageing." *The Journal of Physiology* 594, no. 8 (2016): 2207–22. https://doi.org/10.1113/jphysiol.2014.284513.

Wan, Lixin, Kexin Xu, Yongkun Wei, Jinfang Zhang, Tao Han, Christopher Fry, Zhao Zhang, et al. "Phosphorylation of EZH2 by AMPK Suppresses PRC2 Methyltransferase Activity and Oncogenic Function." *Molecular Cell* 69, no. 2 (January 18, 2018): 279-291.e5. https://doi.org/10.1016/j.molcel.2017.12.024.

Wen, Yuan, Cory M Dungan, C Brooks Mobley, Taylor Valentino, Ferdinand von Walden, and Kevin A Murach. "Nucleus Type-Specific DNA Methylomics Reveals Epigenetic 'Memory' of Prior Adaptation in Skeletal Muscle." *Function* 2, no. 5 (August 28, 2021): zqab038. https://doi.org/10.1093/function/zqab038.

Widmann, Manuel, Andreas M. Nieß, and Barbara Munz. "Physical Exercise and Epigenetic Modifications in Skeletal Muscle." *Sports Medicine* 49, no. 4 (April 2019): 509–23. https://doi.org/10.1007/s40279-019-01070-4.

Wienken, Magdalena, Antje Dickmanns, Alice Nemajerova, Daniela Kramer, Zeynab Najafova, Miriam Weiss, Oleksandra Karpiuk, et al. "MDM2 Associates with Polycomb Repressor Complex 2 and Enhances Stemness-Promoting Chromatin Modifications Independent of P53." *Molecular Cell* 61, no. 1 (January 2016): 68–83. https://doi.org/10.1016/j.molcel.2015.12.008.

Wienken, Magdalena, Ute M. Moll, and Matthias Dobbelstein. "Mdm2 as a Chromatin Modifier." *Journal of Molecular Cell Biology* 9, no. 1 (February 1, 2017): 74–80. https://doi.org/10.1093/jmcb/mjw046.

Williams, Kristine, Germán D. Carrasquilla, Lars Roed Ingerslev, Mette Yde Hochreuter, Svenja Hansson, Nicolas J. Pillon, Ida Donkin, et al. "Epigenetic Rewiring of Skeletal Muscle Enhancers after Exercise

Training Supports a Role in Whole-Body Function and Human Health." *Molecular Metabolism* 53 (July 10, 2021): 101290. https://doi.org/10.1016/j.molmet.2021.101290.

Xu, C., C. D. Fan, and X. Wang. "Regulation of Mdm2 Protein Stability and the P53 Response by NEDD4-1 E3 Ligase." *Oncogene* 34, no. 3 (January 2015): 281–89. https://doi.org/10.1038/onc.2013.557.

Yang, Jer-Yen, Cong S. Zong, Weiya Xia, Hirohito Yamaguchi, Qingqing Ding, Xiaoming Xie, Jing-Yu Lang, et al. "ERK Promotes Tumorigenesis by Inhibiting FOXO3a via MDM2-Mediated Degradation." *Nature Cell Biology* 10, no. 2 (February 2008): 138–48. https://doi.org/10.1038/ncb1676.

Yu, Zheng-Cheng, Yi-Fu Huang, and Sheau-Yann Shieh. "Requirement for Human Mps1/TTK in Oxidative DNA Damage Repair and Cell Survival through MDM2 Phosphorylation." *Nucleic Acids Research* 44, no. 3 (February 18, 2016): 1133–50. https://doi.org/10.1093/nar/gkv1173.

Zhang, Kaijie, Miaomiao Li, Li Yin, Guosheng Fu, and Zhenjie Liu. "Role of Thrombospondin-1 and Thrombospondin-2 in Cardiovascular Diseases (Review)." *International Journal of Molecular Medicine* 45, no. 5 (May 1, 2020): 1275–93. https://doi.org/10.3892/ijmm.2020.4507.

Zhang, Xian, Binkui Li, Abdol Hossein Rezaeian, Xiaohong Xu, Ping-Chieh Chou, Guoxiang Jin, Fei Han, et al. "H3 Ubiquitination by NEDD4 Regulates H3 Acetylation and Tumorigenesis." *Nature Communications* 8, no. 1 (April 2017): 14799. https://doi.org/10.1038/ncomms14799.

Zhou, S., L. Gu, J. He, H. Zhang, and M. Zhou. "MDM2 Regulates Vascular Endothelial Growth Factor MRNA Stabilization in Hypoxia." *Molecular and Cellular Biology* 31, no. 24 (December 15, 2011): 4928–37. https://doi.org/10.1128/MCB.06085-11.