THE EFFECTS OF EXERCISE ON LINE-1 REGULATION

By

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ABSTRACT

Transposable elements (TEs) are mobile DNA and constitute approximately half of the human genome. LINE-1 (L1) is the only active autonomous TE in the mammalian genome and has been implicated, either directly or indirectly, in a number of diseases as well as aging. We have previously reported that L1 expression is lower in skeletal muscle after both acute and chronic resistance training in college-aged males. Building on these findings, we used a rodent model of exercise to better dissect the effects of exercise on skeletal muscle L1 regulation. High running female Wistar rats (n=11 per group) were either given access to a running wheel (EX) or not (SED) at 5 weeks of age, and these conditions were maintained until the rats reached 27 weeks of age. Thereafter mixed gastrocnemius tissue was harvested and analyzed for L1 mRNA expression and DNA content along with other markers involved with L1 regulation. We observed significantly ($p<0.05$) lower L1 mRNA expression, higher L1 DNA methylation, and less L1 DNA in euchromatin in EX versus SED rats. We followed these experiments with *in vitro* drug treatments, which served to mimic exercise-specific signaling events. We found that 4mM 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), which increases AMPK signaling, decreased L1 mRNA expression in L6 myotubes. Our results suggest that long-term voluntary wheel running has the ability to downregulate L1 mRNA via increased DNA methylation and chromatin changes, and these phenomena may be mediated through AMPK signaling.

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CHAPTER I

INTRODUCTION

Transposable elements (TEs), also known as mobile DNA or “jumping genes”, are defined as genetic sequences that have the ability to move from one genomic location to another and are found in almost every eukaryotic organism investigated to date (213). Discovered in the late 1940’s by Barbara McClintock (142), TEs have been investigated in numerous contexts such as their role in gene regulation, drivers in evolution, and more recently as disease-causing agents. The important role that TEs currently play or have played in genetics are enumerable. Coupled with the fact that new and fascinating discoveries are being made in relation to TE biology at an almost astounding pace, the research into TEs is open to fruitful investigations from various fields and disciplines with direct implications to human health and disease (89, 110, 192).

TEs are generally classified into two distinct classes: DNA transposons and retrotransposons, each with their own unique properties and impact on the genome. DNA transposons are defined as genetic elements that can be relocated from one genomic location to another via a mechanism termed “cut and paste”. In contrast, retrotransposons do not relocate per se, but amplify in number via a “copy and paste” mechanism (213); both mechanisms are discussed further below.

The ability of these genes to move about the genome can have significant consequences on the host organism, both positive and negative (89, 117, 149, 169). While it may be easy to recognize the opportunity of these genes to cause mutations, and while this has been shown in

prior literature, the context of these mutations are important to keep in mind. In fact, many mutations caused by TEs can have regulatory functions and have been shown to be adopted by host organisms, a process termed co-option (20, 21, 56, 189, 198). Indeed, there are mutations that are caused by TEs that are detrimental to the host genome. In humans, specific cases of Hemophelia and Duchenne's muscular dystrophy have been caused by a specific TE called Long Interspersed Nuclear Element 1 (LINE-1 or L1) (81). Moreover, various cancers have been associated with an increase in L1 gene expression, or an alteration in L1 promoter methylation. Although these associations have not been shown to be causative, it does suggest a possible relationship between cancerous mutations and L1 (81, 97, 227).

TEs, including L1, have been shown to be responsive to various stimuli and conditions (32–34, 146). For example, in aging, De Cecco et al. observed an increase in L1 mRNA expression and DNA copy number in both skeletal muscle and liver of mice (33). In the same study, when animals were calorie restricted, they showed a significant decrease in both skeletal muscle L1 mRNA expression and L1 DNA copy number. In another model of aging, Van Meter et al. showed that L1 expression in the liver of aged mice was largely controlled by the NAD^+ dependent histone deacetylase SIRT6 (146). In these mice, L1 expression was higher when SIRT6 was quantitatively lower at genomic L1 loci, measured using chromatin immunoprecipitation (ChIP). The authors found that this decrease in SIRT6 at L1 sites coincided with an increase in SIRT6 at DNA damage sites. This finding suggests that L1 regulation may be either directly or indirectly associated with DNA damage responses along with the possibility that L1 may be under metabolic control, given that the SIRT enzymes have been shown to be

sensitive to NAD^+ concentrations (51, 212). Using a more extreme example, in rats that had been dosed with methamphetamine, L1 expression increased concomitantly with a decrease in L1 promoter methylation, a well-researched mechanism in controlling L1 (152). These examples aim to highlight the dynamic nature of L1 regulation, mechanisms of which are still not well understood, even with an abundance of literature on the topic (60, 133, 198, 220).

Physical activity, such as exercise, has shown to be a potent stimulus for adaptation due to the systemic effects that can occur after a single bout of exercise (13, 53, 54). Indeed, exercise that requires large amounts of muscle mass such as aerobic training or resistance training (RT) can stimulate adaptive responses from skeletal muscle, bone, as well as the cardiorespiratory system (70, 84). Responses to exercise requires the orchestration of various signaling pathways that include nutrient signaling, mechanotransduction, and metabolic responses (9, 54, 102, 210). Previous data from our laboratory has shown that exercise may have the ability to regulate L1 in skeletal muscle, with the proposed mechanism owing to an alteration in methylation status at the L1 promoter (180). Multiple labs have shown that both endurance and resistance exercise can alter the methylation status at various genes, however, the ability of exercise in the control of TEs has yet to be explored (12, 13, 122, 187). The goal of this study is to investigate the mechanisms related to how exercise regulates L1 elements using a rodent model. In our model, rats that either exercised for approximately 5 months in early life or remained sedentary during that time frame will be compared for gastrocnemius muscle L1 pathway markers. Additionally, we will perform follow-up experiments using L6 myotubes in an attempt to discover how exercise potentially affects L1 pathway markers.

CHAPTER II

REVIEW OF LITERATURE

Initial Discovery of TEs and Impact

In McClintock's first reports of TEs, she discussed her observations and the nature of the Ac/Ds loci. A loci in which Ac is an autonomous "mutable locus", while the Ds locus required the autonomous Ac for mutability (143–145). This observation was highlighted with an experiment in which McClintock crossed maize plants until they were without the Ac element. When the Ac locus was absent, Ds transposition was not detectable (143). In these experiments, it was noted that either Ac or Ds elements could transpose proximal to a gene and this would have the same effect as though it had inserted into a gene. The ability for these genes to affect gene expression points to their attractiveness as regulative agents, a speculation that did not go unnoticed by McClintock and one that other researchers were keen on as well. Moreover, in her 1950 article, McClintock opined that the observations that were made during her experiments needed multiple published works to fully explain their impact and the initial paper was a general overview of what she was observing (143). Indeed, even today, there is still much to learn regarding TEs and their impact on the genome.

Shortly after McClintock published her reports, Britten and Davidson published their model on gene regulation and how repetitive elements impact the process of gene regulation over evolutionary time (20, 21, 45). They highlighted the high copy number of TEs as well as their ability to be acted upon by various mutation mechanisms that could be at play, providing

evolution with a plethora of DNA sequences that could be co-opted by organisms and selected for as evolutionary time went on (20). Moreover, Britten and Davidson argued that repetitive sequences, interspersed within and between non-repetitive DNA, would provide coding DNA with the sequences necessary to confer regulatory control (20, 21). In support of this, it has been well noted and established that various TEs house their own regulatory units (i.e. gene promoters) that aid in their expression (68, 195, 197, 207, 220). Indeed, various publications have shown that different cis-regulatory sequences (i.e. gene promoters/enhancers) have been co-opted from TEs including L1 (2, 80, 85, 130, 169, 182, 196). Moreover, many TE sequences that have yet to be confirmed as regulatory sequences contain both histone and DNA modifications that normally correlate with cis-regulatory elements (85). These examples provide evidence that TEs make up an important regulatory role within an organism and are not simply "junk DNA" (228).

TEs in Various Organisms

Since the initial discovery of the Ac/Ds TE unit in maize by Barbara McClintock, the TE research field has provided a wealth of knowledge in regards to evolutionary biology, gene regulation, organismal development, and more recently health and disease (23, 69, 80, 208). Interestingly, the TE field has provided a 'pool' of ideas that fuel teleological discussions regarding how TEs evolved and their involvement in complex organisms given their long reach into almost all kingdoms of life (228). Indeed, the TE field is vast and varied with multiple classifications, origins, and roles in one's favorite 'flavor' of organism.

Plants have long been the model organism of choice for researchers in the TE field given plants high genome content of TEs. For example, McClintock's research was based on the maize genome, which owes ~80% of its genome to TE content (186). More recently, many mechanisms regarding retrotransposon activity has been worked out using the *E. coli* model organism. In fact, the mechanism of retrotransposition (amplification using an RNA intermediate) was discovered in 1985 by Boeke et al. using Ty elements, which is an LTR retrotransposon found in *E. coli* (16).

Moving to more complex organisms, *Drosophila melanogaster* has also been instrumental in terms of TE research and discovery, especially in terms of aging and TEs along with regulatory functions (35, 55, 128). The utility of this model organism has been demonstrated from the original heritability studies done by Thomas Morgan's laboratory (151, 175).

With respect to mammalian TEs, mice have become the model organism of choice given their general popularity in research (6, 160). Moreover, transgenic mice have been created that overexpress L1 as a means to investigate the dynamics of L1 in various contexts (160). The creation of transgenic L1 mice coincided with an inducible plasmid of L1 that has been used in multiple settings in order to better understand L1 dynamics, primarily used in cell culture models for mechanistic purposes (79, 118, 150). The Boeke, Moran, and Kazazian laboratories have been pioneers in terms of modern discovery of L1 biology and its impact on human health.

While mice have been the go-to animal model, rats have also been used as a means to study both evolutionary dynamics of L1 and as a model organism for L1 control albeit with less

popularity (43, 68, 112, 135). The Furano laboratory utilized rat samples as a means to investigate L1 evolution and control throughout the 1980's, making seminal discoveries in L1 sequence structure and conservation as well as using L1 elements as a way to track evolutionary dynamics between mammals, using the term "molecular fossils" (17, 69, 123, 159, 163, 164). It is worth noting here that there are inherent differences between rodent genome contents of L1. While rats have been reported to have an L1 genome content of 22%, L1 in the genome of the mouse constitutes ~19%, while L1 makes up ~17% of the human genome (37, 74, 124, 199, 216). These percentages appear to be quite similar, although mice have significantly more estimated functional copies of L1 (~3,000), which could make translating rodent research to humans a little more difficult, especially considering the fact that both rodent genomes contain more active copies of L1 than humans, which is estimated to be ~100 (10, 23, 110, 124, 174).

Given the fact that the LINE family of TEs is found throughout vertebrate genomes along with increasing experimental technology and knowledge in regards to TE biology, it is not hard to imagine that comparative genomics will reveal species and organismal specific mechanisms that could lead to better insight into gene regulation and evolution. This knowledge not only aids research in the aforementioned areas, but also has the ability to provide fertile research fields for health and disease.

Classification of Transposable Elements (TEs) – add other TE classes

Since the seminal discovery of TEs by Barbara McClintock (142), a wealth of knowledge of how TEs impact evolution and genome regulation have come to light (59, 100, 110, 121, 149,

153). At present, TEs are estimated to constitute ~50% of the human genome (124) with some estimations reaching as high as 70% (114). L1 specifically is estimated to make up ~17% of the genome (124). Other mammalian species such as mice and rats also contain large amounts of TEs with DNA content estimations reaching ~40% (37, 74). While it is theorized that these "jumping genes" provide substrates for evolutionary adaptation (65), TEs also provide ample opportunity for mutagenesis to occur (111). Moreover, various diseases have been associated with alterations in TE control (111). In particular, L1 has shown to cause disease in specific cases as indicated above, which includes Duchenne Muscular Dystrophy, Hemophilia A, and various cases of breast and colon cancer (81, 104, 110), as well as showing an increase in expression in association with a slew of other diseases (57, 81). Originally thought to only occur during embryogenesis (107), recent investigations have revisited these initial findings to show that TEs are both expressed and transpose in somatic tissues (14, 117), with a majority of these data in relation to L1. These findings have implications for general genetic control in individual cells, a topic that was discussed not only in McClintock's original work, but also work by developmental biologists Britten and Davidson (20, 21, 45, 166, 198). Britten and Davidson, specifically, noticed the opportunities that TEs afford cells with respect to altering their gene regulatory networks, providing new binding sites for transcription factors, acting as regulatory elements, and creating new protein coding sequences (133, 197, 198, 209, 220). While tempting, these ideas put forth by McClintock and Davidson, as well as others, have been and continue to be debated (91, 194, 198). Given TEs can act as evolutionary substrate they also come with the inherent potential to cause genomic instability within these cells, possibly leading to harmful

genetic mutations (111). While there is evidence to support the statements presented above, ongoing investigation is still taking place regarding active transposition in somatic and nondividing cells, including the functional role that TEs may or may not play in these cells.

Transposable elements fall into two general classes: class I or retrotransposons and class II also known as DNA transposons (62, 213). This classification is based on how TEs move about the genome: retrotransposons utilize an RNA intermediate during their transposition process (or retrotransposition when referring to retrotransposons) (16) while DNA transposons do not, instead they transpose by direct movement of the TE from one location to another via an encoded protein (213). TEs can be subdivided and further classified based on their insertion mechanism, replication strategy, DNA sequence conservation, and insertion location (213). Some popular and widely researched subdivisions include non-long terminal repeat (non-LTR) retrotransposons (i.e. L1), LTR retrotransposons (i.e. endogenous retroviruses), and non-autonomous retrotransposons (ALU and SINEs). Although not the focus of this work, these sub classifications have direct impact on the human genome and are being actively researched today. While the human genome contains thousands of TEs, a specific retrotransposon, L1, has garnered the most attention as of late, possibly due to L1's association with various diseases (25, 26, 75, 201).

LINE-1 Biology

Long Interspersed Nuclear Element-1 (LINE-1 or L1) is a class I autonomous retrotransposon within the LINE order of TEs (213). The term autonomous refers to the ability of

L1 to encode for proteins that allow for its retrotransposition (or amplification) without the need of other protein interactions (213). A fully functional L1 element is 6 kb in length and contains a 5' untranslated region (UTR) that houses an RNA polymerase II promoter, an antisense promoter, encodes for two distinct protein open reading frames (ORF) termed ORF1 and ORF2, has a 3' UTR and is followed by a adenine rich tail (poly A tail) that is variable in length (1, 109, 161). Within the 3' UTR is an important diagnostic set of nucleotides. In humans, the 3' UTR contains a trinucleotide (5'-ACA-3') that is used to determine the younger more active LINEs versus the older and retrotransposition incompetent LINEs (174). Although there is estimated to be >500,000 copies of L1 elements, only a fraction of those elements retain the ability to retrotranspose, also called 'hot' L1s, with the non-functional elements acting as a 'molecular fossil' record of old L1 elements and families (23). In contrast, while most of the L1 elements cannot retrotranspose, some of them have been co-opted by cells for various other functions, which include acting as gene enhancers and DNA binding sites (66, 130). Given the sheer volume of genetic material that L1 constitutes (~17% of our genome), it does not seem too far-fetched that L1 may serve multiple roles that are still yet to be discovered (228).

L1 elements encode for two proteins; Open Reading Frame 1 protein and Open Reading Frame 2 protein (ORF1p and ORF2p, respectively) (67, 68, 216). Both proteins are encoded within the same mRNA, a regulatory mechanism reminiscent of simpler eukaryotes and not found regularly in mammals (174). The ORF proteins have distinct properties with ORF1p acting as an RNA binding protein and ORF2p having both endonuclease and reverse transcriptase capabilities (26, 41, 162). The L1 ORFs also show distinct translational control with some data

indicating that L1 undergoes translation via internal ribosomal entry sites, similar to viruses, while other data indicate a more traditional AUG start codon along with the 5' methyl cap (3, 50). Nevertheless, the concentration of L1 proteins appears to be different for each ORF, which is not surprising given the fact that translation of the L1 mRNA happens via a re-initiation mechanism for the more downstream ORF (ORF2) (86, 87).

A general overview of L1 amplification begins with the expression of a functional L1 element (23, 115). Multiple TFs have been shown to activate L1 expression in various context and sometimes within specific tissues (8, 40, 137, 155). Once L1 is expressed, it is transported out of the nucleus and translated into two distinct proteins; ORF1p and ORF2p. Again, due to being a bicistronic mRNA, L1 has been reported to show translational mechanisms that resemble viruses compared to eukaryotic genes. However, there are investigations that report contrasting data, as indicated above. Nevertheless, once translated, the ORF proteins show a cis-preference for L1 mRNA (118). ORF1p and ORF2p show unique binding properties in that ORF1p appears to bind in trimers throughout the length of the L1 mRNA, possibly acting to protect the mRNA from nuclease digestion, while ORF2p seems to primarily bind toward the 3' end of an L1 mRNA, creating an L1 ribonucleoprotein (RNP) complex. Once the RNP complex is formed, it can translocate back into the nucleus whereby ORF2p will utilize its endonuclease enzyme activity to nick DNA, liberating a 3' OH group that acts as a primer for the reverse transcriptase activity of ORF2p. The L1 mRNA is then reverse transcribed by ORF2p and the cDNA that is created is inserted into the genome. While the exact mechanisms for this pathway are still

currently under investigation, this general 'script' is thought to be the sequence of events that leads to L1 amplification (39, 119, 147, 203).

L1 is unique in the human genome in the sense that it is an autonomous retrotransposon; that is the L1 elements encode for all the proteins that are needed for its transposition and it is generally accepted that it is currently the only autonomous TE in mammals (213). There are also reports to suggest that L1 can also be influenced by signaling cascades and extracellular signals such as MAPK/ERK signaling (39). Although a cis-preference has been shown for the L1 ORF proteins and the L1 mRNA, other TEs, such as ALU elements and SINEs, can "hijack" the L1 machinery in order to perpetuate their own amplification throughout the human genome (167, 174, 195).

Methods to Detect L1

A method for L1 detection in mammalian cells was developed in 1996 by Moran et al. in which an L1 is transfected into a cell culture (150). The transfected L1 contains an indicator cassette, a neomycin gene, which is expressed only after retrotransposition. This retrotransposition-specific gene expression is possible due to the fact that the neomycin cassette is inserted backwards, separated by a sense oriented intron. The synthetic L1 also contains its own promoter along with a poly-adenylation signal (150). This ensures that neomycin will not be expressed unless the L1 is reverse transcribed and the intron is removed. This assay allows for two different validation steps: a) one could easily probe for neomycin expression from the cultured cells, and b) the cells could be plated and tested for antibiotic resistance.

The plasmid developed by Moran et al. has since been advanced, adding an inducible promoter that is responsive to doxycycline (160). This allows for specific timing of L1 construct expression that is in the control of the researcher. Furthermore, the same laboratory developed a mouse model that contained the L1 inducible construct in order provide proof of principle experiments and to investigate L1 overexpression in terms of an observable phenotype (160).

While overexpression is a novel method that allows for mechanistic and phenotypic observations, the expression of endogenous L1 and its consequences has been understandably difficult. With so many copies throughout the genome, accurate readings of these elements pose a unique challenge (5). If you also consider the fact that modern microarray and next-generation sequencing technologies used to measure gene expression primarily utilize methods that discard repetitive reads, the problem of recording accurate L1 expression becomes that much more daunting (15, 76, 126). In terms of human gene expression studies, multiple laboratories, including the ones mentioned above, have developed bioinformatic techniques (i.e. unique RNA-seq scripts/pipelines) that allow for accurate L1 gene expression analysis (75, 196, 199, 201, 211). One problem, however, is that these methods are primarily limited to either human or mouse L1 expression. As mentioned above, although rodents and other mammals contain L1, their sequence similarity, regulation, copy number, and specific phenotypic context can vary by species. These shortcomings are currently being investigated, which will allow for greater insight into L1 control and associated mechanisms.

L1 Regulation and Control

Cells have various mechanisms at work to silence TEs, some of which are highly investigated mechanisms and pathways such as DNA methylation and histone modifications (22, 133, 220). Moreover, organisms also use RNA interference (RNAi), which include transcriptional silencing, post transcriptional silencing, and heterochromatin formation that may or may not include the aforementioned mechanisms (139, 225). While these methods are quite effective at keeping TEs repressed, these control systems can be disrupted. Disrupting the silencing mechanisms of cells can lead to aberrant gene expression from TEs and this has shown to lead to increased L1 DNA content as well as other retrotransposon integration, such as ALUs and SINEs, as mentioned above (23, 32, 33, 117, 147).

Hypothesis on Epigenetics and TE 'Control'

It was noted very early on in TE research that there were mechanisms to keep TE expression at a minimum (224). A general working hypothesis was formed in that cells were working to keep TEs "under control" by using modifications on DNA, which was repressing their transcription and therefore their ability to transpose or retrotranspose and that these epigenetic processes had evolved for this very reason (224). Given the shear amount of methylated DNA at TEs, it seems entirely reasonable to reach this conclusion. This argument is further strengthened by the fact that repressive histone marks such as H3K27me3 and H3K9me3 are also found in high abundance within TE genes (98, 139). Taken together, these data seem to support the hypothesis that epigenetic signaling evolved in order to "protect" the organism, however, there are more recent data that is questioning this hypothesis. By comparing the

evolution of TEs as compared to epigenetic signaling and prokaryotic genomes, a number of articles have argued that TEs coevolved with their eukaryotic hosts either with or before these elaborate epigenetic mechanisms (60, 228). This directly questions the validity of the hypothesis that epigenetic signaling evolved to control these detrimental DNA segments. These data are incredibly difficult to interpret, which may explain why these questions have not been easily investigated. Notwithstanding, the fact that TEs are epigenetically controlled is hard to argue (133, 139, 148, 168, 197).

DNA Methylation and TEs

DNA Methylation is a DNA modification that is associated with changes in gene expression (103). Cells express both methylation and demethylation enzymes that work to help regulate genes by either depositing a methyl group to cytosine nucleotides (methylation enzyme) or by removing methyl groups from cytosine nucleotides (demethylation enzymes) (127). Methylation at gene promoters is a well-studied modification that is associated with the repression of gene expression. This DNA modification is primarily deposited on the fifth carbon on cytosine residues that are followed by a guanine nucleotide, termed CpG islands (88, 103, 176). It was noted very early on that methylation took place at TEs and that methylation of the TEs correlated with gene repression (224). Methylation of cytosine residues also offers a DNA modification that can be recognized by a variety of transcription factors and binding proteins that also help to repress gene expression such as HP1 or MeCP2, although cytosine methylation within gene bodies and elsewhere may actually help promote transcription (83, 136, 127, 154).

DNA demethylation can occur via active or passive processes (127). Active demethylation occurs via enzymes such as the Ten-eleven translocation (TET) enzymes, while passive demethylation mainly occurs during processes such as DNA replication where methyl marks are not deposited on the newly synthesized DNA strand. Both appear to be physiologically important and seem to occur during specific time points and contexts (136).

The TET family of proteins are dioxygenases that use molecular oxygen to catalyze the DNA demethylation reaction (127, 219). The TET family is made up of the proteins TET1, TET2, and TET3 that appear to have some tissue specificity as well as a temporal component to their expression (47, 48, 226). Interestingly, TETs utilize alpha-ketoglutarate (aKG) as a substrate and produce succinate as an inhibitory product (206, 219, 222, 226), two metabolic intermediates that have shown to be altered in various contexts including disease and exercise (44, 72, 73, 93, 140). Furthermore, recent data suggests that TETs may be regulated by signaling pathways such as AMPK signaling, making them an attractive target for metabolic and exercise related investigations (31, 222).

Histone Modifications and TEs

As briefly discussed above, TEs are controlled by both DNA and histone modifications. While DNA modifications are fairly straight forward, histone modifications appear to have their own "code" (141, 193). Histone marks can be transcriptionally activating, repressive, and it has also been noted that so called "bivalent" histone marks exists whereby histones have both

repressive and activating modifications on them, making interpreting these data quite difficult and rather nuanced (36, 129, 200).

Current data regarding histone modifications have focused on histone methylation and histone acetylation; marks that are deposited by histone methyltransferases or histone acetyl transferases, respectively (4). Other histone marks exist, such as phosphorylation, arginylation, and succinylation. However, with respect to TEs, methylation and acetylation are the most often cited (85, 96, 106, 170, 200). The deposition of histone marks are catalyzed by specific protein complexes that are typically recruited by transcription factors or by proteins that bind in specific circumstances. Examples include the muscle specific transcription factor MyoD that when bound to specific DNA sequences (E-Boxes) recruits the histone acetyltransferase complex P300, resulting in acetylated histones (29, 185). Ultimately, this results in a euchromatic environment and the transcriptional machinery now has access to muscle specific genes. An example of repressive complex recruitment would start with DNA methylation, in which a protein, MECP2, that primarily binds methylated DNA, then recruits histone methylation machinery and histone deacetylation complexes to promote heterochromatin formation and the repression of gene transcription, An observation that has been made in regards to L1 (154). Moreover, a recent report has shown that L1 is also controlled via histone deacetylation, specifically catalyzed by the NAD^+ dependent histone deacetylase SIRT6 (146). The authors found that during aging or with excessive DNA damage, SIRT6 was titrated away from L1 elements and found at higher concentrations at the DNA damage sites. Intriguingly, it has been suggested that SIRT enzymes can act as “metabolic sensors” due to the fact that they utilize NAD^+ as a cofactor, an electron

carrier used during both glycolysis and the TCA cycle (51, 58, 77, 146, 202, 212). These studies highlight a possible relationship between TEs, and L1 specifically, with cellular metabolism, especially given the fact that disease as well as exercise has shown to modify NAD^+ metabolism (19, 64, 101, 113, 212). In regards to metabolic inputs, a substantial amount of data supports the hypothesis that metabolism influence chromatin modifications independent of and including SIRT activity (42, 131, 171). For example, histone demethylases utilize either FAD^+ or aKG as substrates for the demethylation reaction, two molecules that are involved in electron transfer in the mitochondria and the TCA cycle, respectively (105, 156). In disease models, it has also been shown that these molecules can be limiting similar to SIRTs, although their role in normal physiology is not quite clear (171, 184).

RNAi and TEs

Besides the silencing mechanisms discussed above, eukaryotes utilize other means as well in order to silence or control TEs, specifically RNA interfering pathways or RNAi. RNAi can be used either transcriptionally or post-transcriptionally (139). When used transcriptionally, RNA generated from the gene that is to be silenced is used as a targeting nucleotide sequence (90). Termed RNA Induced Transcriptional Silencing (RITS), the RNA generated binds with specific proteins such as DICER and AGO1, that will process and bind to the nucleotide sequence. AGO1 will then use the nucleotide sequence to find complementary nascent RNA transcripts, wherein once a complimentary transcript is found, histone modification enzymes such as histone methyltransferase and histone deacetylases are recruited in order to form

heterochromatin (24). While this mechanism is prevalent in various plant species, mammals do not encode some of the proteins used in this pathway such as RNA dependent RNA polymerase. A variation of this RNAi is used, but utilizes PIWI proteins and has only so far been characterized in the germline (158, 172).

Both mechanisms, those found in plants and mammals, work to silence TEs including L1. Although RNAi in plants is decently understood, RNAi via RITS in mammals, mechanistically, is largely unknown. Interestingly, it was recently shown that RNAi, in the germ cells of male mice, was needed in order to silence L1 retrotransposition (158). The specific RNAi mechanism, termed the piRNA pathway, helps to re-establish DNA methylation at L1 elements shortly after fertilization. Inability to do this, via transgenic mice deficient in a specific piRNA pathway protein, results in increased L1 retrotransposition and ultimately the death of these germ cells (158). This study suggests that uncontrolled L1 retrotransposition may be detrimental to cells, a hypothesis that has been suggested in other disciplines as well (32–34, 49, 78, 97, 217).

L1 in Various Contexts

L1 in Development and Reprogramming

The role and functions of L1 in a cell during various states and conditions has recently been a hot topic of discussion. No doubt due to various findings and associations in medical research along with developmental and evolutionary biology (110, 165). In regards to development, L1and other TEs are upregulated during specific developmental stages, more than likely due to the "wiping" of DNA methylation during early stages of development (191). This

loss of methylation removes the repressive transcriptional signal and allows various transcriptional activators access to L1 DNA (218, 221). This allows for an increase L1 expression and possibly increased retrotransposition. Interestingly, this increase in L1 expression may serve a need within a developing embryo. Recently, Percharde et al. showed that when L1 is knocked down via antisense oligonucleotides, developing cells could not progress to a new cell state, specifically moving from the 2 cell state to the 4 cell state (165). Furthermore, the authors showed that L1 was necessary for transcription of 4 cell state specific genes and increased ribosomal RNA, suggesting that L1 helps to increase growth signals during these specific stages (165). They related the mechanism to that of a long non coding RNA, which can be used to attract transcriptional machinery to genes of interest via chromatin looping, a common mechanism in transcription of DNA (185). In line with these findings, the reprogramming of cells to that of embryonic or pluripotent stem cells also shows an increase in L1 expression (71, 99, 215). This may be due to the regulation mentioned above as well as the fact that multiple binding sites exist for transcription factors that are typically found in these specific cell states (8, 121, 137).

Gene Regulation

L1, along with other TEs, has been hypothesized to be involved in gene regulation, a sentiment put forth by Barbara McClintock among others (20, 21, 45, 145, 198). These regulatory mechanisms were suggested to be most likely due to the fact that L1 houses an endogenous gene promoter that is amplified along with the L1 ORFs (68). This would allow for

the amplification of new binding sites throughout the genome, working to “rewire” or alter the regulation of various genes (198). Moreover, the amplification of L1 elements could also work to change the chromatin landscape by recruiting various chromatin modifying complexes such as histone methyltransferases, among others, that could also be at play in terms of gene regulation (18, 108, 120). Given the structural similarity between promoters and enhancers, it also appears plausible that L1 promoters could be co-opted as gene enhancers, regulatory elements that aid in gene expression from distant sites (7, 28, 30, 132, 173, 177, 194, 207). These are just a few examples of how TEs, and L1 specifically, could work to alter gene regulation. The data are by no means one directional and more investigations are needed to help uncover how L1 is involved in gene regulation both in stem cells and somatic tissues.

L1 in Physiology and Disease

While not always the driving factor for the respective disease, altered TE expression is associated with over 100 different diseases and has been mechanistically tied to a number of specific cases of those disease, such as DMD, hemophilia, and colorectal cancer (82). Moreover, there are other interesting observations in which TE regulation is altered, but the physiological outcome of these alterations has yet to be established. In one example in which heat stress is applied, the yeast species S. *Pombe* show a significant increase in TE expression, interestingly with a transposition preference for heat response genes (61). In rats, it was shown that methamphetamine ingestion increases L1 expression in the rat brain as well as a decrease in methylation at the L1 promoter (152). In regards to humans, a recent paper has shown that L1

promoter methylation is lower in individuals that have high BMIs compared to those with normal BMIs (138). These examples highlight the malleable regulatory nature of TEs although their physiological significance has yet to be elucidated.

Along with being associated with a slew of diseases, L1 expression is also seen to increase with age that is accompanied by an increase in L1 integration (33). This has been shown in multiple models systems that include *drosophila,* mouse, and human cells (32–35). Moreover, L1 expression has been hypothesized by some researchers to be involved in the aging process, possibly by causing mutations in somatic tissues due to retrotransposition, possibly by altering regulatory landscapes or by directly inserting into protein coding genes (30, 32, 33, 125). This is an attractive hypothesis with data to suggest a direct involvement of L1, although an exact mechanism is still under investigation. While not exhaustive, these examples highlight how TE regulation is not static and responds to environmental stimulus. The physiological consequences of increased or decreased L1 expression will be an interesting area of research going forward.

Exercise as a Means to Regulate L1

Exercise can affect almost every tissue in the body, depending on the type and intensity of the exercise bout (63). Furthermore, some of the pioneers in bioenergetics used skeletal muscle as a model system in order to study how energy, stored in the form of ATP, is both consumed and generated (116). Exercise is also used as a model in order to investigate the mechanisms of skeletal muscle hypertrophy as well as oxygen kinetics and it has also been shown to be beneficial as an intervention for a number of diseases as well as a means for helping

to prevent certain diseases such as type II diabetes (9, 11, 38, 63, 157, 214). These data point to a role for metabolic control in both health and disease.

With respect to exercise, it was recently reported that resistance training (RT) directly affects the epigenetic status of skeletal muscle, creating a 'memory' of passed training events that may aid in the adaptation process when a subsequent stimulus is encountered (187, 188). Previous data has also shown that after acute bouts of exercise, DNA methylation at exercise responsive genes is decreased, accompanied by a concomitant increase in gene expression including PGC1α and PPARδ, among others (12, 13). Moreover, we recently published a study that wherein individuals that underwent acute and chronic bouts of RT had a significant reduction in L1 gene expression and an increase in L1 promoter methylation (180). These reports may provide a wealth of new knowledge in epigenetic control as well as how humans adapt, using exercise as a model system. What this also highlights is a novel control system of L1 that exercise may influence, possibly due to alterations in metabolic control either during or after training (72, 73, 93).

In the last decade, a plethora of research has suggested and supports a role for metabolism in epigenetic control (27, 42, 95, 134, 171, 223). These include, but are not limited to, post-translational modifications of histone proteins such as methylation and acetylation as well as DNA methylation. Indeed, there are more than 60 different post-translational modifications, some of which are influenced by metabolism, and many of which have not been assigned function (200). Investigated model systems include various stem cell populations such as pluripotent stem cells, muscle stem cells, multiple tissues including skeletal muscle and liver,

suggesting that metabolic inputs affect epigenetic signals across different tissues and cell types (52, 92, 94, 181, 183, 190, 204). As mentioned above, process such as histone methylation and acetylation have been suggested to be regulated by metabolic intermediates. These data highlight and suggest that metabolic alterations have an influence over chromatin modifications, influencing various aspects of gene regulation.

Given the data presented above along with the data collected from our laboratory, the goal of the present study is to better characterize the mechanisms at play that we believe are working to regulate L1 gene expression with exercise. Our hypotheses are that the control of L1 is independent of exercise type as our previous data has shown that L1 methylation is sensitive to RT, and that the observations of L1 expression alterations are dependent on exercise. To test these hypotheses, we will analyze skeletal muscle from rodents that exercised via voluntary wheel running and compare them to strain-matched sedentary control animals. We expect to see a decrease in L1 mRNA expression, measured via qPCR using primers that were specifically designed for certain L1 copies as well as an increase of DNA methylation at the L1 promoter using those same PCR primers. Although DNA methylation at the L1 promoter is a final outcome measure, we also aim to investigate whether this increase in methylation is due to an increase in enzyme activity of DNMTs or possibly due to a decrease in DNA demethylation enzyme activity (i.e. TETs). In order to test this, we will compare overall DNMT enzyme activity between groups as well as overall SIRT enzyme activity given there is data that supports a role for SIRT6 in L1 gene repression. Following *in-vivo* data analysis, cell culture will be used as an exploratory model in order to better characterize *in-vivo* findings. Using L6 myotubes, we

will also have the ability to focus on elucidating exercise specific signals that may be altering L1 gene expression by mimicking AMPK activation via AICAR and increased calcium concentration via caffeine. These methods will aid in the understanding of how L1 is regulated and specifically how exercise can alter the expression of these retrotransposons.

CHAPTER III

METHODS

Animals

All live animal experiments and dissections occurred at the University of Missouri, and these experimental procedures were approved by the Institution's Animal Care and Use Committee. For the present study, muscle was procured and analyzed by our research group at Auburn University.

Rats used were from a previous study in which high voluntary wheel running (HVR) rats were selected and bred as described earlier (178, 179, 205). Briefly, female Wistar rats that were selectively bred for high levels of wheel running were divided into two groups based on whether they did or did not have access to a voluntary running wheel (EX and SED, respectively). Both groups were weaned at 28 days of age. EX rats (n=11) were provided access to running wheels from 5 weeks of age until the end of the experiment when they were 27 weeks of age, whereas SED rats (n=11) were housed in standard cages without running wheels until 27 weeks of age. Rats were provided drinking water and standard rodent chow *ad libitum*. Data such as VO_2 peak and running distances have been previously reported (205). On the day of dissections animals were euthanized using CO_2 asphyxiation in the afternoon hours during the middle of the light cycle (1400-1800); gastrocnemius muscles were rapidly removed, and muscles were flash-frozen in liquid N_2 and stored at -80°C until being shipped to Auburn University.

Tissue preparation for protein analyses

Muscles were removed from -80ºC storage; tissue was crushed on a liquid nitrogen-cooled ceramic mortar and pestle, and approximately 50 mg of tissue from each rodent was placed in 7 mL glass vials containing 500 µL of ice-cold cell lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na-EDTA, 1 mM EGTA, 1% Triton, 20 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 µg/mL leupeptin] (Cell Signaling; Danvers, MA, USA). Tissues were homogenized via glass dounce homegenization and homogenates were centrifuged at 12,000 *g* for 10 min. Supernatants were transferred to 1.7 mL tubes, and protein concentration was determined using a BCA assay (Thermo Scientific, Waltham, MA, USA). Supernatants were subsequently prepared for SDS-PAGE using 4x Laemmli buffer at 2 µg/µL for Western blot analysis, and 15 µL were loaded onto 4-15% SDS-polyacrylamide pre-casted gels obtained from (Bio-Rad Laboratories; Hercules, CA, USA). 1x SDS-PAGE run buffer (Ameresco; Framingham, MA, USA) was used for electrophoresis at 180 V for 60 min. Thereafter, proteins were transferred via 200 mA constant current for 120 min to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were then stained with Ponceau S and digital images were captured using a gel documentation system (UVP, Upland, CA, USA) to ensure equal loading of samples among lanes. Membranes were then blocked at room temperature with 5% nonfat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST) for one hour. All of the following primary antibodies were incubated overnight at 4ºC in a solution of TBST containing 5% bovine serum albumin (BSA; Ameresco): rabbit anti-cytochrome C (1:1000; Genetex, cat# GTX108585), mouse anti-ORF1p (1:1000;

Abcam, cat# ab76726), or rabbit anti-phosphorylated AMPKα (Thr172) (1:1000; CST, cat #2531). The following day, membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (1:2000; Cell Signaling cat# 7076 and 7074, respectively) in a solution of TBST containing 5% BSA at room temperature for one hour. Thereafter, membranes were developed using an enhanced chemiluminescent reagent (Luminata Forte HRP substrate; EMD Millipore, Billerica, MA, USA) with band densitometry subsequently assessed by use of a digital gel documentation system and associated densitometry software (UVP, Upland, CA). Densitometry on white band values for each aforementioned target was normalized to a corresponding dark band using Ponceau densitometry values. Additionally, values were normalized to SED group to yield relative protein expression levels.

RNA isolation and qPCR prep

Approximately 20 mg of powdered frozen gastrocnemius muscle from each rodent were placed in 500 µL of Ribozol (Ameresco, Solon, OH, USA) per the manufacturer's recommendations. Thereafter, phase separation was achieved according to manufacturer's instructions for RNA isolation. Following RNA precipitation and pelleting, pellets were resuspended in 20 µl of RNase-free water, and RNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA). 10 µg of isolated RNA was DNase treated (Turbo DNase, cat# AM2238, Invitrogen, Carlsbad, CA, USA) and cDNA (2 µg) was synthesized using a commercial qScript™ cDNA SuperMix

(Quanta Biosciences, Gaithersburg, MD, USA) per the manufacturer's recommendations. qPCR was performed with gene-specific primers and SYBR-green-based methods (Quanta Biosciences), in a real-time PCR thermal cycler (Bio-Rad, Hercules, CA, USA). The final volume of qPCR reactions were 20 µl, which contained a final concentation of 2 µm of forward and reverse primers and 25 ng of cDNA. All reactions were performed in duplicate. Primers for housekeeping genes (Table 1) were designed with primer designer software (Primer3Plus, Cambridge, MA, USA), and melt curve analyses demonstrated that one PCR product was amplified per reaction for all reactions. For rats, two primer sets were designed to interrogate L1 mRNA expression based on previous data (112). The first primer set (L1-3) was designed to probe for the most active LINE-1, while the second primer set (L1-Tot) was designed to encompass all full-length LINE-1 elements including those that contained a 5' promoter, but did not have the ability to undergo retrotransposition based on mutations throughout the L1 gene. The L1-3 and L1-Tot primers were designed for the 5' end of L1 elements based on a previous study characterizing functional elements (112). A diagram of general primer location is depicted in Figure 1a. The forward and reverse primer sequences for all genes are listed in Table 1. Fold-change values from the SED rats were performed using the $2^{\Delta\Delta Cq}$ method where $2^{\Delta Cq}$ = 2^[housekeeping gene (HKG) Cq – gene of interest Cq], and $2^{\Delta\Delta Cq}$ (or fold-change) = [$2^{\Delta Cq}$ value/$2^{\Delta Cq}$ average of SED group]. The geometric mean of housekeeping genes (*Fbl, Ppia, Hprt)* was used to normalize mRNA expression results. There were no between-group differences in the geometric mean of housekeeping genes.

Table 1. Rat primer sequences used for real-time qPCR

Gene	Accession number/L1 Family
(Rat) Fibrillarin (*Fbl*; HKG) FP (5’ → 3’): CTGCGGAATGGAGGACACTT RP (5’ → 3’): GATGCAAACACAGCCTCTGC	NM_001025643.1
(Rat) Beta-2 microglobulin (*B2m*; HKG) FP (5’ → 3’): GGAAACTGAGGGGAGTAGGG RP (5’ → 3’): CCTGGGCTTTCATCCTAACA	NC_005102.4
(Rat) Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; HKG) FP (5’ → 3’): TGATGCCCCCATGTTTGTGA RP (5’ → 3’): GGCATGGACTGTGGTCATGA	NC_005103.4
(Rat) Cyclophilin A (*Ppia*; HKG) FP (5’ → 3’): GCATACAGGTCCTGGCATCT RP (5’ → 3’): AGCCACTCAGTCTTGGCAGT	NM_017101.1
(Rat) Hypoxanthine phosphoribosyltransferse 1 (*Hprt*; HKG) FP (5’ → 3’): AAGACAGCGGCAAGTTGAAT RP (5’ → 3’): GGGCCTGTGTCTTGAGTTCA	NM_012583.2
(Rat) L1 (L1-3) FP (5’ → 3’): GACCATCTGGAACCCTGGTG RP (5’ → 3’): GGGCCTGTGTCTTGAGTTCA	DQ100473.1
(Rat) L1 (L1-Tot) FP (5’ → 3’): GGAAGAGACCACCAACACTG RP (5’ → 3’): GAAGGTTTAGCTCTCCCTCC	DQ100473.1 DQ100475.1 DQ100476.1 DQ100477.1 DQ100474.1 DQ100482.1

Legend: HKG, housekeeping gene; bp, base pairs; UTR, untranslated regions.

DNA isolation and qPCR prep

Powdered gastrocnemius muscle was removed from -80º C storage and approximately 15 mg was processed using the commercially available DNeasy Blood & Tissue Kit (QIAGEN,

Venlo, Netherlands) per the manufacturer's recommendations including RNase treatment. Following DNA precipitation and pelleting, DNA was eluted with 100 µL of elution buffer from the kit per manufacturer's recommendations, and DNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA). Isolated DNA was then used for downstream assays described below as well as L1 DNA content using qPCR using the L1-3 and L1-Tot qPCR primers.

DNA methylation analysis

L1 promoter methylation analysis was performed on isolated gastrocnemius DNA (described above) using a commercially available methylated DNA immunoprecipitation (MeDIP) kit (Abcam, Cambridge, MA, USA). Prior to performing the MeDIP assay, 1.5 µg of gastrocnemius DNA was digested using MseI due to the fact that this enzyme did not digest DNA within our qPCR primer sequences (New England BioLabs, Ipswich, MA, USA). Following digestion reactions, 1 µg of DNA was used for immunoprecipitation with an anti-5-methylcytosine antibody provided within the kit. qPCR was then performed on the methylated DNA enriched sample using the L1-3 and L1-Tot primers listed above given both primer pairs span CpG-rich areas in the 5' UTR. Additionally, 0.1 µg of residual input DNA from each sample was used as a control in a parallel reaction in order to normalize qPCR results. Both the experimental and control wells contained 25 ng of DNA for the reactions and were carried out using the same primer- and SYBR green-based methods as described above for qPCR. Fold-

change scores in L1 DNA methylation were calculated as follows: a) $2^{\Delta Cq}$ values were calculated whereby ΔCq = input DNA Cq – methylated DNA Cq, and b) fold-change values were then obtained by dividing each individual $2^{\Delta Cq}$ value by the SED $2^{\Delta Cq}$ group mean.

L1 chromatin accessibility analysis

LINE-1 chromatin accessibility was assessed from each rodent using a commercially available kit (Chromatin Accessibility Assay Kit, product #: ab185901; Abcam) per the manufacturer's recommendations. Due to sample limitations, only a subset of animals were analyzed in this experiment (SED, n = 10; EX, n = 9). Briefly, methods involved obtaining DNA, digesting the DNA using a proprietary nuclear digestion buffer, and performing qPCR on digested versus undigested samples. The premise of the assay is that a gene of interest localized to euchromatin regions is more susceptible to digestion and, thus, possesses a lower qPCR amplification signal relative to genes in heterochromatin regions. Gastrocnemius DNA was freshly isolated using propriety columned based methods provided by kit. qPCR was performed as described above on 25 ng of digested DNA using the L1-3 and L1-Tot primers to decipher fold-change in genomic L1 DNA residing in euchromatin. Undigested DNA from each sample (25 ng) was used as a control to normalize RT-PCR results. Fold-change in L1-3 heterochromatin DNA was calculated using the $2^{\Delta\Delta Cq}$ method where $2^{\Delta Cq}$ = 2^[undigested L1-3 DNA Cq – digested L1-3 DNA Cq] and $2^{\Delta\Delta Cq}$ (or fold-change) = [$2^{\Delta Cq}$ value/$2^{\Delta Cq}$ average of SED group].

Nuclear DNMT activity assay

Prior to assaying nuclear DNA methyltransferase (DNMT) activity, nuclear protein extraction was performed on frozen gastrocnemius muscle (~25 mg) using a commercially-available kit (Nuclear Extraction Kit; Abcam cat #ab113474) per the manufacturer's recommendations. Global DNMT activity of nuclear isolates (10 µL) was assessed using a commercially available kit (DNMT Activity Assay Kit; Abcam cat# ab113467) per the manufacturer's recommendations. Briefly, gastrocnemius tissue was homogenized in an extraction buffer provided by the kit, followed by centrifugation at 12,000 RPM for 15 min. Supernatant was then discarded followed by the addition of a dithiothreitol solution and protease inhibitor cocktail to the aforementioned extraction buffer, all provided in the kit. The newly made solution was added to the pellet, incubated on ice (15 min) while vortexing every 3 min. The suspension was centrifuged at 14,000 RPM, the supernatant was transferred to a new tube, and protein content was determined using the BCA method described above. DNMT activity was measured on the nuclear extracts. Reactions were carried out on a 96-well plate that included Adomet substrate (provided in the kit) and 10 µg of nuclear extract followed by incubation at 37° C. Wells were then washed and capture antibody was added to the wells and incubated at room temperature. Wells were washed again and detection antibody was then added followed by another incubation at room temperature for 30 minutes. Wells were washed again followed by the addition of enhancer solution and a 30 minute incubation time at room temperature. Wells were washed, a developer solution was added and wells are incubated for 10 minutes away from light at room temperature followed by the addition of a stop solution. Wells were then read in a

microplate reader at 450 nm for 10 minutes. DNMT activity is expressed as relative expression units (REU), which are normalized to input muscle weights.

Citrate synthase activity assay

Citrate Synthase (CS) activity was performed as previously described by our laboratory (151). The assay principle is based upon the reduction of 5,50-dithiobis (2- nitrobenzoic acid) (DTNB) at 412 nm (extinction coefficient 13.6 mmol/L/cm) coupled to the reduction of acetyl-CoA by the CS reaction in the presence of oxaloacetate. Briefly, 2 µg of skeletal muscle protein was added to a mixture composed of 0.125 mol/L Tris–HCl (pH 8.0), 0.03 mmol/L acetyl-CoA, and 0.1 mmol/L DTNB. The reaction was initiated by the addition of 5 µL of 50 mmol/L oxaloacetate and the absorbance change was recorded for 1 min.

mRNA microarray

Total RNA was extracted from the gastrocnemius muscle as described above and a subset of samples from each group (n=8 per group) were shipped to a commercial service for transcriptome-wide analysis (Thermo Fisher Scientific). Briefly, RNA integrity was first assessed using microfluidic gel electrophoresis. Thereafter, RNA was subjected to first and second strand cDNA synthesis reactions. A series of reactions was then used to generate fragmented, single-stranded cDNA which was labelled using the WT Terminal Labeling Kit.

Labelled cDNA was then hybridized on Rat Clariom S array chips according to the manufacturer's instructions. Differential gene expression was calculated using Transcriptome Analysis Console 4.0, summarization was performed using the SST-RMA algorithm, and results were provided as tab-delimited files. Log_2 signal intensity values for mRNAs related to DNA methylation were then calculated and statistically compared via independent samples t-test between SED and EX rats. DNMT and TET Genes were chosen based on their ability to regulate L1 elements in prior literature (46, 154, 173).

Cell culture

For all *in vitro* experiments, rat L6 myoblasts (passage 2) were grown in growth medium (DMEM, 10% FBS, 1% penicillin/streptomycin, and 0.1% gentamycin) on 12-well plates at a seeding density of 2 x 10^5 per mL under standard culture conditions (37°C in a 5% CO_2 atmosphere). Once myoblast growth reached 80–90% confluency, differentiation was induced by removing growth medium and replacing it with differentiation medium [DM; DMEM, 2% (vol/vol) horse serum, 1% penicillin/streptomycin, and 0.1% gentamycin]. DM was then replaced every 24 h for 6 days to allow for myotube growth.

For drug screening experiments in L6 myotubes, cells were treated (n = 4 per condition) with one of six treatments, or vehicle, in order to investigate the possible exercise-associated mechanisms that regulate L1 gene expression (Table 2).

Table 2. Drug treatments for *in vitro* screening

Treatment	Mechanism of action	Concentration
DMSO (control)	Vehicle for all drugs; served as control	0.1%
5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR)	Increase AMPK activity	1 mM
Caffeine (Caf)	Increase intracellular calcium release	5 mM
Rotenone (Rot)	Increase intracellular NADH levels	100 nM
Resveratrol (Res)	Increase SIRT activity	10 μM
Trichostatin A (TA)	Decrease HDAC activity	100 nM
5-Azacytidine (5-AZA)	Decrease DNMT activity	10 μM

Treatments included AICAR (1 mM; Enzo, Farmingdale, NY, USA, cat# BML-EI330), caffeine (5 mM; Alfa Aesar, Ward Hill, Massachusetts, cat# A10431), rotenone (100 nM; Enzo, Farmingdale, NY, USA, cat# ALX350360G001), resveratrol (10 μM; TCI America, Portland, OR, cat# R0071),the global HDAC inhibitor trichostatin A (100 nM; Promega, Madison, WI, cat# G656A), 5-Azacytidine (5-AC) (10 μM; TCI America, Portland, OR, USA) or 0.1% DMSO (Corning Inc., Corning, NY, cat# 25-950-CQC) as a vehicle control. All conditions contained the same amount of DMSO in their final concentrations. Treatments were added to fresh DM when DM was replaced, and treatment DM was left on cells for a 3-h incubation. After 3 h of treatment, cells were washed once with PBS and lysed with Ribozol for RNA isolation as described above. Following RNA isolation, qPCR was performed as described above to interrogate L1 mRNA expression using the SYBR green-based qPCR methods and the rat primers described above.

Follow-up AICAR experiments in L6 myotubes (n = 6 wells per dose) were performed as described above with the exception of different doses of AICAR (1 mM, 2 mM, and 4 mM) being applied to cells over a 3-h treatment period.

Statistics

Statistics were performed using SPSS v 23.0 (IBM, Armonk, NY, USA). Prior to statistical analyses, assumptions testing was performed on all dependent variables. In cases where data were not normally distributed we applied either $\log_{10}$ or square root transformations. Dependent variables were analyzed using independent samples t-tests. For data that violated Levene's Test for Equality of Variances a Welch's t-test was used. For *in vitro* data analysis, dependent variables were analyzed using independent samples t-tests relative to the vehicle condition. For data that were not normally distributed after transformation we performed nonparametric statistics (Mann-Whitney U Test). Additionally, Pearson product correlations were also performed on LINE-1 expression and select dependent variables. Magnitude of effects are expressed in the results using Cohen's d calculations and effect sizes of 0.2, 0.5, and 0.8 were considered small, moderate, and large, respectively. Statistical significance for all null hypothesis testing was set at $p<0.05$. All data are presented as mean ± standard deviation (SD).

CHAPTER IV

COMPLETED MANUSCRIPT (to be submitted to AJP Cell Physiol in July, 2019)

Five months of voluntary wheel running downregulates skeletal muscle LINE-1 activity in rats

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ABSTRACT

Transposable elements (TEs) are mobile DNA and constitute approximately half of the human genome. LINE-1 (L1) is the only active autonomous TE in the mammalian genome and has been implicated, either directly or indirectly, in a number of diseases as well as aging. We have previously reported that L1 expression is lower in skeletal muscle after both acute and chronic resistance training in college-aged males. Building on these findings, we used a rodent model of exercise to better dissect the effects of exercise on skeletal muscle L1 regulation. Intrinsically high running female Wistar rats (n=11 per group) were either given access to a running wheel (EX) or not (SED) at 5 weeks of age, and these conditions were maintained until the rats reached 27 weeks of age. Thereafter mixed gastrocnemius tissue was harvested and analyzed for L1 mRNA expression and DNA content along with other markers involved with L1 regulation. We observed significantly ($p<0.05$) lower L1 mRNA expression, higher L1 DNA methylation, and less L1 DNA in accessible chromatin regions in EX versus SED rats. We followed these experiments with *in vitro* drug treatments in L6 myotubes, which served to mimic exercise-specific signaling events. We found that 4mM 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), which increases AMPK signaling, decreased L1 mRNA expression in L6 myotubes. Our results suggest that long-term voluntary wheel running has the ability to downregulate L1 mRNA via increased DNA methylation and chromatin changes, and these phenomena may be mediated through AMPK signaling.

Keywords: LINE-1, L1, Methylation, Retrotransposons, Exercise

INTRODUCTION

Transposable elements (TEs), often referred to as mobile DNA or “jumping genes”, are genetic elements that have the ability to change their position in a genome (5, 41). There are numerous types of TEs that exist based on their mechanism of transposition (or movement) and are categorized into two distinct classes: DNA transposons and retrotransposons (61). The former uses a DNA intermediate termed a “cut and paste” mechanism, while the latter utilizes a “copy and paste mechanism” where an RNA intermediate is reverse transcribed into cDNA prior to integration into the genome resulting in an amplification of the retrotransposon (61).

Long INterspersed Element-1 (LINE-1, L1) is one of the most abundant TEs in humans and constitutes large portions of the genome of rats and mice as well with estimations of 17%, 18%, and 21%, respectively, significantly outnumbering protein coding genes (~2%) (10, 21, 36, 52). L1 is the only autonomous retrotransposon active in mammalian genomes, meaning it encodes for all the machinery needed for its retrotransposition (for review see (5, 32, 52)). L1 is a 6 kilobase genetic element that contains an internal RNA polymerase II (Pol II) promoter within the 5’ untranslated region. L1 encodes for two different proteins: Open Reading Frame 1 protein (ORF1p), an RNA binding protein, and Open Reading Frame 2 protein (ORF2p), which has both endonuclease and reverse transcriptase activities. The first step of L1 retrotransposition is the recruitment of RNA Pol II to its internal promoter followed by transcription of the L1 DNA into a bicistronic mRNA – meaning both ORF proteins are encoded within the same mRNA. L1 mRNA is then translated into its respective proteins, and the L1 proteins

preferentially bind to their mRNA forming an L1 ribonucleoprotein (L1RNP) complex. L1RNP then translocates to the nucleus, where ORF2p catalyzes the nicking of DNA at consensus TTAAAA target sites, followed by reverse transcription L1 mRNA into cDNA by ORF2p, ultimately leading to the *de novo* integration of L1 cDNA into the genome resulting in target site duplications on both the 5' and 3' ends of the newly inserted gene (11, 35, 46, 59). It is also worth noting that recent elegant genetic studies have shed light on the amplification mechanisms and site selection of L1 (see (16, 58)). While L1 ORF proteins show *cis* preference for L1 transcripts, other mRNAs and TEs can also bind with the ORF proteins ultimately leading to amplification of those genes and TEs, respectively (52). Other TEs such as Short INterspersed Elements (SINEs) can "hijack" L1 machinery, evidenced by the number of SINEs in the human genome (~1,000,000 copies) (52). The relevance and potential physiological impact of these amplifications are detailed elsewhere (9, 48).

L1, collectively with other TEs, has had "positive" implications during evolution. For example, there is evidence that the DNA binding domain of the DNA transposon Tc1/mariner has been co-opted by animals in the PAX3 gene, which is important for skeletal muscle development (28, 47). Another example is that L1 may be involved in a quality control mechanism in oogenesis by which defective oocytes are removed (18, 39). More recently, TEs, and L1 specifically, have been implicated in a number of diseases (23, 32). These include both associative as well as causative examples such as increased L1 expression in various cancers and direct insertions causing specific cases of Duchenne's muscular dystrophy (14, 24, 25, 29, 45).

With respect to TE and L1 research, skeletal muscle has only been highlighted in a handful of studies (2, 34, 38). A seminal paper by De Cecco et al. reported that skeletal muscle and liver L1 mRNA and DNA levels are significantly higher in older mice (36 months of age) versus younger mice (5 months of age), and the authors speculated that increased tissue L1 activity may accelerate the aging process (8). In line with these findings, Sirt6 has been shown to act as a repressor of L1 expression, but Sirt6-mediated L1 repression is lost in aged mice (42). Interestingly, the same laboratory demonstrated that muscle mass loss was attenuated in Sirt6 knockout mice that were treated with reverse transcriptase drugs which inhibit ORF2p enzyme activities (56).

Our laboratory has previously shown that skeletal muscle L1 mRNA expression was significantly decreased while L1 DNA methylation was increased in college-aged males after both acute and chronic resistance training (RT) (55). Further, De Cecco et al. reported that skeletal muscle and liver L1 mRNA levels were significantly lower in lifelong calorically-restricted mice compared to *ad libitum* fed counterparts (8). While preliminary, these data highlight the possibility that exercise and caloric restriction may down-regulate tissue L1 expression through a conserved mechanism. Given the data presented above, the goal of the present study was to examine how long-term voluntary wheel running affects skeletal muscle L1 markers in rats. Additionally, we sought to determine potential mechansims through which exercise may operate to regulate L1 gene expression. We hypothesized that long-term exercise would down-regulate skeletal muscle L1 markers, although we had no a priori hypothesis regarding how this mechanistically occurs. Consistent with our previous results in humans, we

find that long-term endurance training through voluntary wheel running, down-regulates skeletal muscle L1 expression and increases L1 DNA methylation. Further, we provide *in vitro* evidence in L6 myotubes that these phenomena may occur through enhanced AMP-activated protein kinase (AMPK) signaling.

MATERIALS AND METHODS

Animals

All live animal experiments and dissections occurred at the University of Missouri, and these experimental procedures were approved by the Institution's Animal Care and Use Committee. For the present study, muscle was procured and analyzed by our research group at Auburn University.

Rats used were from a previous study in which high voluntary wheel running (HVR) rats were selected and bred as described earlier (53, 54, 60). Briefly, female Wistar rats that were selectively bred for high levels of wheel running were divided into two groups based on whether they did or did not have access to a voluntary running wheel (EX and SED, respectively). Both groups were weaned at 28 days of age. EX rats (n=11) were provided access to running wheels from 5 weeks of age until the end of the experiment when they were 27 weeks of age, whereas SED rats (n=11) were housed in standard cages without running wheels until 27 weeks of age. Rats were provided drinking water and standard rodent chow *ad libitum*. Data such as VO_2 peak

and running distances have been previously reported (60). On the day of dissections animals were euthanized using CO_2 asphyxiation in the afternoon hours during the middle of the light cycle (1400-1800); gastrocnemius muscles were rapidly removed, and muscles were flash-frozen in liquid N_2 and stored at -80°C until being shipped to Auburn University.

Tissue preparation for protein analyses

Muscles were removed from -80ºC storage; tissue was crushed on a liquid nitrogen-cooled ceramic mortar and pestle, and approximately 50 mg of tissue from each rodent was placed in 7 mL glass vials containing 500 µL of ice-cold cell lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na-EDTA, 1 mM EGTA, 1% Triton, 20 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 µg/mL leupeptin] (Cell Signaling; Danvers, MA, USA). Tissues were homogenized via glass dounce homegenization and homogenates were centrifuged at 12,000 *g* for 10 min. Supernatants were transferred to 1.7 mL tubes, and protein concentration was determined using a BCA assay (Thermo Scientific, Waltham, MA, USA). Supernatants were subsequently prepared for SDS-PAGE using 4x Laemmli buffer at 2 µg/µL for Western blot analysis, and 15 µL were loaded onto 4-15% SDS-polyacrylamide pre-casted gels obtained from (Bio-Rad Laboratories; Hercules, CA, USA). 1x SDS-PAGE run buffer (Ameresco; Framingham, MA, USA) was used for electrophoresis at 180 V for 60 min. Thereafter, proteins were transferred via 200 mA constant current for 120 min to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were then stained

with Ponceau S and digital images were captured using a gel documentation system (UVP, Upland, CA, USA) to ensure equal loading of samples among lanes. Membranes were then blocked at room temperature with 5% nonfat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST) for one hour. All of the following primary antibodies were incubated overnight at 4ºC in a solution of TBST containing 5% bovine serum albumin (BSA; Ameresco): rabbit anti-cytochrome C (1:1000; Genetex, cat# GTX108585), mouse anti-ORF1p (1:1000; Abcam, cat# ab76726), or rabbit anti-phosphorylated AMPKα (Thr172) (1:1000; CST, cat #2531). The following day, membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (1:2000; Cell Signaling cat# 7076 and 7074, respectively) in a solution of TBST containing 5% BSA at room temperature for one hour. Thereafter, membranes were developed using an enhanced chemiluminescent reagent (Luminata Forte HRP substrate; EMD Millipore, Billerica, MA, USA) with band densitometry subsequently assessed by use of a digital gel documentation system and associated densitometry software (UVP, Upland, CA). Densitometry on white band values for each aforementioned target was normalized to a corresponding dark band using Ponceau densitometry values. Additionally, values were normalized to SED group to yield relative protein expression levels.

RNA isolation and qPCR prep

Approximately 20 mg of powdered frozen gastrocnemius muscle from each rodent were placed in 500 µL of Ribozol (Ameresco, Solon, OH, USA) per the manufacturer's

recommendations. Thereafter, phase separation was achieved according to manufacturer's instructions for RNA isolation. Following RNA precipitation and pelleting, pellets were resuspended in 20 μl of RNase-free water, and RNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA). 10 μg of isolated RNA was DNase treated (Turbo DNase, cat# AM2238, Invitrogen, Carlsbad, CA, USA) and cDNA (2 μg) was synthesized using a commercial qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA) per the manufacturer's recommendations. qPCR was performed with gene-specific primers and SYBR-green-based methods (Quanta Biosciences), in a real-time PCR thermal cycler (Bio-Rad, Hercules, CA, USA). The final volume of qPCR reactions were 20 μl, which contained a final concentation of 2 μm of forward and reverse primers and 25 ng of cDNA. All reactions were performed in duplicate. Primers for housekeeping genes (Table 1) were designed with primer designer software (Primer3Plus, Cambridge, MA, USA), and melt curve analyses demonstrated that one PCR product was amplified per reaction for all reactions. For rats, two primer sets were designed to interrogate L1 mRNA expression based on previous data (33). The first primer set (L1-3) was designed to probe for the most active LINE-1, while the second primer set (L1-Tot) was designed to encompass all full-length LINE-1 elements including those that contained a 5' promoter, but did not have the ability to undergo retrotransposition based on mutations throughout the L1 gene. The L1-3 and L1-Tot primers were designed for the 5' end of L1 elements based on a previous study characterizing functional elements (33). A diagram of general primer location is depicted in Figure 1a. The forward and reverse primer sequences for all genes are listed in Table 1. Fold-

change values from the SED rats were performed using the $2^{\Delta\Delta Cq}$ method where $2^{\Delta Cq}$ = 2^[housekeeping gene (HKG) Cq – gene of interest Cq], and $2^{\Delta\Delta Cq}$ (or fold-change) = [$2^{\Delta Cq}$ value/$2^{\Delta Cq}$ average of SED group]. The geometric mean of housekeeping genes (*Fbl, Ppia, Hprt)* was used to normalize mRNA expression results for the *in vivo* data while the geometric mean of *B2m, Ppia, and Hprt* were used for *in vitro* data. There were no between-group differences in the geometric mean of housekeeping genes.

[***INSERT TABLE 1 HERE***]

DNA isolation and qPCR prep

Powdered gastrocnemius muscle was removed from -80ºC storage and approximately 15 mg was processed using the commercially available DNeasy Blood & Tissue Kit (QIAGEN, Venlo, Netherlands) per the manufacturer's recommendations including RNase treatment. Following DNA precipitation and pelleting, DNA was eluted with 100 µL of elution buffer from the kit per manufacturer's recommendations, and DNA concentrations were determined in duplicate at an absorbance of 260 nm by using a NanoDrop Lite (Thermo Scientific, Waltham, MA, USA). Isolated DNA was then used for downstream assays described below as well as L1 DNA content using qPCR using the L1-3 and L1-Tot qPCR primers.

DNA methylation analysis

L1 promoter methylation analysis was performed on isolated gastrocnemius DNA (described above) using a commercially available methylated DNA immunoprecipitation (MeDIP) kit (Abcam, Cambridge, MA, USA). Prior to performing the MeDIP assay, 1.5 µg of gastrocnemius DNA was digested using MseI due to the fact that this enzyme did not digest DNA within our qPCR primer sequences (New England BioLabs, Ipswich, MA, USA). Following digestion reactions, 1 µg of DNA was used for immunoprecipitation with an anti-5-methylcytosine antibody provided within the kit. qPCR was then performed on the methylated DNA enriched sample using the L1-3 and L1-Tot primers listed above given both primer pairs span CpG-rich areas in the 5' UTR. Additionally, 0.1 µg of residual input DNA from each sample was used as a control in a parallel reaction in order to normalize qPCR results. Both the experimental and control wells contained 25 ng of DNA for the reactions and were carried out using the same primer- and SYBR green-based methods as described above for qPCR. Fold-change scores in L1 DNA methylation were calculated as follows: a) $2^{\Delta Cq}$ values were calculated whereby ΔCq = input DNA Cq – methylated DNA Cq, and b) fold-change values were then obtained by dividing each individual $2^{\Delta Cq}$ value by the SED $2^{\Delta Cq}$ group mean.

L1 chromatin accessibility analysis

LINE-1 chromatin accessibility was assessed from each rodent using a commercially available kit (Chromatin Accessibility Assay Kit, product #: ab185901; Abcam) per the

manufacturer's recommendations. Due to sample limitations, only a subset of animals were analyzed in this experiment (SED, n = 10; EX, n = 9). Briefly, methods involved obtaining DNA, digesting the DNA using a proprietary nuclear digestion buffer, and performing qPCR on digested versus undigested samples. The premise of the assay is that a gene of interest localized to euchromatin regions is more susceptible to digestion and, thus, possesses a lower qPCR amplification signal relative to genes in heterochromatin regions. Gastrocnemius DNA was freshly isolated using propriety columned based methods provided by kit. qPCR was performed as described above on 25 ng of digested DNA using the L1-3 and L1-Tot primers to decipher fold-change in genomic L1 DNA residing in euchromatin. Undigested DNA from each sample (25 ng) was used as a control to normalize RT-PCR results. Fold-change in L1-3 heterochromatin DNA was calculated using the $2^{\Delta\Delta Cq}$ method where $2^{\Delta Cq}$ = 2^[undigested L1-3 DNA Cq – digested L1-3 DNA Cq] and $2^{\Delta\Delta Cq}$ (or fold-change) = [$2^{\Delta Cq}$ value/$2^{\Delta Cq}$ average of SED group].

Nuclear DNMT activity assay

Prior to assaying nuclear DNA methyltransferase (DNMT) activity, nuclear protein extraction was performed on frozen gastrocnemius muscle (~25 mg) using a commercially-available kit (Nuclear Extraction Kit; Abcam cat #ab113474) per the manufacturer's recommendations. Global DNMT activity of nuclear isolates (10 µL) was assessed using a commercially available kit (DNMT Activity Assay Kit; Abcam cat# ab113467) per the

manufacturer's recommendations. Briefly, gastrocnemius tissue was homogenized in an extraction buffer provided by the kit, followed by centrifugation at 12,000 RPM for 15 min. Supernatant was then discarded followed by the addition of a dithiothreitol solution and protease inhibitor cocktail to the aforementioned extraction buffer, all provided in the kit. The newly made solution was added to the pellet, incubated on ice (15 min) while vortexing every 3 min. The suspension was centrifuged at 14,000 RPM, the supernatant was transferred to a new tube, and protein content was determined using the BCA method described above. DNMT activity was measured on the nuclear extracts. Reactions were carried out on a 96-well plate that included Adomet substrate (provided in the kit) and 10 µg of nuclear extract followed by incubation at 37° C. Wells were then washed and capture antibody was added to the wells and incubated at room temperature. Wells were washed again and detection antibody was then added followed by another incubation at room temperature for 30 minutes. Wells were washed again followed by the addition of enhancer solution and a 30 minute incubation time at room temperature. Wells were washed, a developer solution was added and wells are incubated for 10 minutes away from light at room temperature followed by the addition of a stop solution. Wells were then read in a microplate reader at 450 nm for 10 minutes. DNMT activity is expressed as relative expression units (REU), which are normalized to input muscle weights.

Citrate synthase activity assay

Citrate Synthase (CS) activity was performed as previously described by our laboratory (2, 24). The assay principle is based upon the reduction of 5,50-dithiobis (2- nitrobenzoic acid) (DTNB) at 412 nm (extinction coefficient 13.6 mmol/L/cm) coupled to the reduction of acetyl-CoA by the CS reaction in the presence of oxaloacetate. Briefly, 2 µg of skeletal muscle protein was added to a mixture composed of 0.125 mol/L Tris–HCl (pH 8.0), 0.03 mmol/L acetyl-CoA, and 0.1 mmol/L DTNB. The reaction was initiated by the addition of 5 µL of 50 mmol/L oxaloacetate and the absorbance change was recorded for 1 min.

mRNA microarray

Total RNA was extracted from the gastrocnemius muscle as described above and a subset of samples from each group (n=8 per group) were shipped to a commercial service for transcriptome-wide analysis (Thermo Fisher Scientific). Briefly, RNA integrity was first assessed using microfluidic gel electrophoresis. Thereafter, RNA was subjected to first and second strand cDNA synthesis reactions. A series of reactions was then used to generate fragmented, single-stranded cDNA which was labelled using the WT Terminal Labeling Kit. Labelled cDNA was then hybridized on Rat Clariom S array chips according to the manufacturer's instructions. Differential gene expression was calculated using Transcriptome Analysis Console 4.0, summarization was performed using the SST-RMA algorithm, and results were provided as tab-delimited files. Log_2 signal intensity values for mRNAs related to DNA methylation were then calculated and statistically compared via independent samples t-test

between SED and EX rats. DNMT and TET genes were chosen based on their ability to regulate L1 elements in prior literature (12, 43, 44, 51).

Cell culture

For all *in vitro* experiments, rat L6 myoblasts (passage 2) were grown in growth medium (DMEM, 10% FBS, 1% penicillin/streptomycin, and 0.1% gentamycin) on 12-well plates at a seeding density of 2 x 10^5 per mL under standard culture conditions (37°C in a 5% CO_2 atmosphere). Once myoblast growth reached 80–90% confluency, differentiation was induced by removing growth medium and replacing it with differentiation medium [DM; DMEM, 2% (vol/vol) horse serum, 1% penicillin/streptomycin, and 0.1% gentamycin]. DM was then replaced every 24 h for 6 days to allow for myotube growth.

For drug screening experiments in L6 myotubes, cells were treated (n = 4 per condition) with one of six treatments, or vehicle, in order to investigate the possible exercise-associated mechanisms that regulate L1 gene expression (Table 2).

[***INSERT TABLE 2 HERE***]

Treatments included AICAR (1 mM; Enzo, Farmingdale, NY, USA, cat# BML-EI330), caffeine (5 mM; Alfa Aesar, Ward Hill, Massachusetts, cat# A10431), rotenone (100 nM; Enzo,

Farmingdale, NY, USA, cat# ALX350360G001), resveratrol (10 µM; TCI America, Portland, OR, cat# R0071),the global HDAC inhibitor trichostatin A (100 nM; Promega, Madison, WI, cat# G656A), 5-Azacytidine (5-AC) (10 µM; TCI America, Portland, OR, USA) or 0.1% DMSO (Corning Inc., Corning, NY, cat# 25-950-CQC) as a vehicle control. All conditions contained the same amount of DMSO in their final concentrations. Treatments were added to fresh DM when DM was replaced, and treatment DM was left on cells for a 3-h incubation. After 3 h of treatment, cells were washed once with PBS and lysed with Ribozol for RNA isolation as described above. Following RNA isolation, qPCR was performed as described above to interrogate L1 mRNA expression using the SYBR green-based qPCR methods and the rat primers described above.

Follow-up AICAR experiments in L6 myotubes (n = 6 wells per dose) were performed as described above with the exception of different doses of AICAR (1 mM, 2 mM, and 4 mM) being applied to cells over a 3-h treatment period.

Statistics

Statistics were performed using SPSS v 23.0 (IBM, Armonk, NY, USA). Prior to statistical analyses, assumptions testing was performed on all dependent variables. In cases where data were not normally distributed we applied either $\log_{10}$ or square root transformations. Dependent variables were analyzed using independent samples t-tests. For data that violated Levene's Test for Equality of Variances a Welch's t-test was used. For *in vitro* data analysis,

dependent variables were analyzed using independent samples t-tests relative to the vehicle condition. For data that were not normally distributed after transformation we performed nonparametric statistics (Mann-Whitney U Test). Additionally, Pearson product correlations were also performed on LINE-1 expression and select dependent variables. Magnitude of effects are expressed in the results using Cohen's d calculations and effect sizes of 0.2, 0.5, and 0.8 were considered small, moderate, and large, respectively. Statistical significance for all null hypothesis testing was set at $p<0.05$. All data are presented as mean ± standard deviation (SD).

RESULTS

L1 DNA content and mRNA expression

One concern when analyzing L1 data is the fact that different animals can have differing amounts of L1 encoded in their genome. There were no significant between-group differences for either L1-Tot or L1-3 DNA ($t(20) = 1.154$, $p = 0.262$, $d = 0.49$ and $t(20) = 0.663$, $p = 0.515$, $d = 0.28$, respectively; Fig. 1b/c). L1-Tot and L1-3 mRNA expression, however, were significantly lower in the EX group ($t(20) = 3.941$, $p = 0.001$, $d = 1.68$ and $t(12) = 2.831$, $p = 0.016$ (Welch's t-test), $d = 1.21$, respectively; Fig. 1d/e). Thus, we posit that lower mRNA levels in EX animals was due to the exercise stimulus and not inherent L1 DNA content between groups. In spite of L1 mRNA differences, there was no significant between-group difference for ORF1 protein levels ($t(20) = 1.009$, $p = 0.325$, $d = 0.43$; Figure 1f). We next wanted to test for potential associations between our markers of exercise training with the expression of the most active L1

gene, L1-3. We observed a significant negative correlation between L1-3 mRNA and CS activity ($p = 0.011$, $r = -0.532$; Figure 1g). Moreover, we observed a similar effect with cytochrome C ($p = 0.036$, $r = -0.448$; Fig. 1h).

[***INSERT FIGURE 1 HERE***]

DNA Methylation and Chromatin Dynamics

With the microarray data, we focused on the mRNA expression of enzymes that regulate DNA methylation given that L1 expression is highly regulated by DNA methylation (12) (Fig. 2a). Interestingly, *Dnmt1* mRNA expression was significantly greater ($t(10) = 3.024$, $p = 0.012$ (Welch's t-test), $d = 1.51$; Fig. 2a) in EX versus SED, and *Tet2* mRNA expression was significantly lower in EX versus SED ($t(14) = 3.544$, $p = 0.003$, $d = 1.77$; Fig. 2a). Neither *Dnmt3a* nor *Dnmt3b* were significantly different between the groups ($t(14) = 0.672$, $p = 0.512$, $d = 0.34$ and $t(14) = 0.232$, $p = 0.820$, $d = 0.12$, respectively). *Tet1* and *Tet3* mRNA was also not significantly different ($t(14) = 0.357$, $p = 0.726$, $d = 0.18$ and $t(14) = 0.623$, $p = 0.543$, $d = 0.32$, respectively).

In order to test whether enzyme activity was different between the groups, we tested global DNMT activity and found that the EX group had significantly higher DNMT activity ($t(20) = 4.039$, $p = 0.001$, $d = 1.72$; Fig. 2b). Interestingly, the L1-3 and L1-Tot primer sets also

indicated that L1 DNA methylation was significantly higher in EX versus SED rats ($t(20) = 2.994$, $p = 0.007$, $d = 1.28$ and $t(20) = 2.478$, $p = 0.022$, $d = 1.06$, respectively; Fig. 2c/d). Based on our microarray, as well as our DNA methylation data, we chose to investigate L1 chromatin accessibility using the L1-3 and L1-Tot primer sets. The EX group had significantly lower amounts of L1-Tot in euchromatin ($t(17) = 2.323$, $p = 0.033$, $d = 1.06$; Fig. 2e), while L1-3 was trending lower in the EX group as well ($t(15) = 1.784$, $p = 0.093$, $d = 0.81$; Fig. 2f).

[***INSERT FIGURE 2 HERE***]

Cell Culture Screen for Exercise Specific Pathways

An *in vitro* screen was used to examine if one or multiple exercise-related signaling mediators could decrease L1 mRNA expression (Fig 3a/b). Relative to vehicle-only treatments, 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), was not significantly different for L1-Tot or L1-3 ($t(6) = 1.439$, $p = 0.200$, $d = 1.02$, $t(6) = 0.661$, $p = 0.533$, $d = 0.47$, respectively). Caffeine was trending upwards for both L1-Tot and L1-3 ($t(6) = 2.279$, $p = 0.063$, $d = 1.61$ and $t(6) = 2.110$, $p = 0.079$, $d = 1.49$, respectively) while no differences were observed for rotenone ($t(6) = 0.625$, $p = 0.555$, $d = 0.44$ and $t(6) = 1.394$, $p = 0.213$, $d = 1.11$, respectively) or resveratrol ($t(6) = 0.199$, $p = 0.849$, $d = 0.14$ and $t(6) = 0.486$, $p = 0.644$, $d = 0.34$, respectively). Trichostatin A trended upwards ($t(6) = 2.238$, $p = 0.067$, $d = 1.58$ and $t(6) = 2.051$, $p = 0.086$, $d =$

1.45, respectively), while 5-Azacytidine was not significantly different ($t(4) = 0.537$, $p = 0.620$ (Welch's t-test), $d = 0.38$ and $t(3) = 0.673$, $p = 0.547$ (Welch's t-test), $d = 0.48$, respectively).

AICAR, which is a known stimulator of the AMP-dependent protein kinase (AMPK) pathway, was the only compound that decreased L1-Tot and L1-3 mRNA expression. Because of this finding, along with the involvement of increased AMPK activity during endurance exercise, we performed follow-up experiments using increasing concentrations of AICAR.

Using treatments of 1mM, 2mM and 4mM concentrations of AICAR in rat L6 myotubes ($t(6) = 6.047$, $p = 0.001$ (Welch's t-test), $d = 3.49$, $t(6) = 27.794$, $p < 0.001$ (Welch's t-test), $d = 16.05$, $t(5) = 17.484$, $p < 0.001$, $d = 10.09$, Fig 3c), we did not observe a significant decrease in L1-Tot or L1-3 at the 1mM dose ($p = 0.423$ (Mann-Whitney U test), $d = 0.93$ and $p = t(10) = 1.501$, $p = 0.164$, $d = 0.87$, respectively) or the 2mM dose ($t(10) = 0.556$, $p = 0.590$, $d = 0.32$ and $p = 0.873$ (Mann-Whitney U test), $d = 0.33$, respectively). We did, however, observe significantly lower L1-3 and L1-Tot mRNA expression in cells that were treated with 4mM of AICAR ($t(10) = 2.423$, $p = 0.036$, $d = 1.40$ and $p = 0.010$ (Mann-Whitney U test), $d = 2.50$, respectively, Fig 3 d/e).

We then sought to examine if DNA methylation was different between the treatment conditions compared to control conditions. Using the same L1 primers, we did not observe a significant difference between the control condition and 1mM of AICAR treatment for L1-Tot or L1-3 ($t(9) = 0.885$, $p = 0.399$, $d = 0.54$ and $t(9) = 0.970$, $p = 0.357$, $d = 0.59$, respectively, Fig 3 f/g). Paradoxical to what we would expect, however, L1 methylation for L1-Tot and L1-3 was

significantly lower in both the 2mM ($t(5) = 2.940$, $p = 0.032$ (Welch's t-test), $d = 1.70$ and $t(5) = 2.981$, $p = 0.030$ (Welch's t-test), $d = 1.72$, respectively) and the 4mM AICAR condition ($t(5) = 2.733$, $p = 0.040$ (Welch's t-test), $d = 1.58$ and $t(5) = 2.797$, $p = 0.036$ (Welch's t-test), $d = 1.61$, respectively).

[***INSERT FIGURE 3 HERE***]

DISCUSSION

As described prior, L1 has the potential to be detrimental to an organism; however, only a handful of papers exist investigating L1 activity in muscle (2, 34, 38). In line with our previous human work, we provide evidence that long-term voluntary wheel running in rats downregulates L1 mRNA expression possibly through observed increases in 5' UTR methylation as well as reducing chromatin accessibility in regions containing L1 DNA. Further, the *in vitro* data add to the current body of literature by suggesting exercise may decrease L1 expression through increased AMPK signaling.

A burgeoning field of research links cellular metabolism to epigenetic control with direct implications *in vivo* such as cell fate decisions and various diseased states (7, 15, 37, 49). Histone acetyltransferases/deacetylases and histone/DNA methyltransferases/demethylases are directly influenced by metabolic intermediates and electron carriers including S-adenosyl methionine

(DNMTs and HMTs), acetyl-coA (HATs), alpha ketoglutarate (TETs, JmjCs), FAD (LSD), and NAD^+ (SIRTs), among others (3, 17, 49). While there are data that support metabolic control of these enzymes in various diseased states resulting in altered gene regulation, whether these mechanisms are involved during or after exercise has yet to be determined. Studies from Gibala et al. showed that skeletal muscle alpha ketoglutarate and succinate concentrations (activating substrate and inhibitory product of the TETs and JmjCs, DNA and histone demethylases, respectively) are altered in skeletal muscle after an intense bout of endurance exercise (3, 17, 19, 20, 49). Taken together, although we did observe transcriptional changes for *Tet2*, and various histone modifiers (data not shown) these speculations would need to be the focus of future research endeavors that would require both genetic and biochemical manipulations with careful considerations being made for *in vivo* K_m values of the aforementioned enzymes *in vivo*.

Given the *in vitro* findings, one exercise-specific pathway that we speculate downregulates L1 mRNA expression is increased AMPK activity. Numerous studies have demonstrated that endurance exercise increases skeletal muscle AMPK activation during and acutely following endurance exercise (30). Additionally, and as mentioned prior, long-term caloric restriction, which is a well-known activator of skeletal muscle AMPK, has been shown to down-regulate L1 mRNA in rodent skeletal muscle (6, 8). Surprisingly, while we did observe lower L1 expression in the 4mM AICAR conditions, this was accompanied with lower L1 methylation, which is a paradoxical finding. There are data suggesting a direct involvement of AMPK with both DNMT and TET enzymes with Marin et al. showing that DNMT1 activity is

inhibited upon phosphyorylation by AMPK while TET2 activity appeared to increase after phosphylation by AMPK as reported by Wu et al. (40, 62).

Although we did not expect the observed results, L1 can be regulated by a variety of mechanisms such as the piwi-interacting RNA pathway or other RNA interfering pathways including microRNAs. It should be noted, however, that these pathways have previously been investigated in terms of mammalian embryos or gametes making the interpretation in skeletal muscle, or somatic tissue in general, more difficult including whether exercise can affect these control mechanisms (4, 50, 57). With these data in mind, it appears that L1 control may be more complex than anticipated in somatic tissues and this potential is ripe for future investigation.

Limitations

An initial limitation is that only a single muscle was analyzed between groups. This can make interpreting results from a single tissue difficult and does not lend itself well to making whole-body inferences. Furthermore, the *in vitro* drug screen for exercise-specific pathways was limited due to the fact that dose-response curves and exposure times were not investigated. In this regard, some of the compounds screened could possibly have an effect on L1 expression if different doses or exposure times were used. For example, caffeine showed a surprising result (trended toward increasing L1-Tot and L1-3 mRNA expression), although we chose not to pursue caffeine in downstream analyses. Finally, it is possible that the effects of voluntary exercise were not fully manifested because the rats were genetically predisposed for exercise.

Even with these limitations, our data suggest that "exercise signals" in skeletal muscle have the ability to alter transcriptional control of L1, and future research is needed in order to investigate if/how this is related to skeletal muscle physiology.

Conclusions

This is the first report demonstrating that long-term voluntary wheel running downregulates skeletal muscle L1 gene expression. We speculate that this may occur through changes in L1 5'UTR methylation and chromatin accessibility as well as increased AMPK activity. However, these two mechanisms may operate independently to down-regulate L1 expression.

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TABLES AND FIGURES WITH LEGENDS

Table 1. Rat primer sequences used for real-time qPCR

Gene	Accession number/L1 Family
(Rat) Fibrillarin (*Fbl*; HKG) FP (5' → 3'): CTGCGGAATGGAGGACACTT RP (5' → 3'): GATGCAAACACAGCCTCTGC	NM_001025643.1
(Rat) Beta-2 microglobulin (*B2m*; HKG) FP (5' → 3'): GGAAACTGAGGGGAGTAGGG RP (5' → 3'): CCTGGGCTTTCATCCTAACA	NC_005102.4
(Rat) Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; HKG) FP (5' → 3'): TGATGCCCCCATGTTTGTGA RP (5' → 3'): GGCATGGACTGTGGTCATGA	NC_005103.4
(Rat) Cyclophilin A (*Ppia*; HKG) FP (5' → 3'): GCATACAGGTCCTGGCATCT RP (5' → 3'): AGCCACTCAGTCTTGGCAGT	NM_017101.1
(Rat) Hypoxanthine phosphoribosyltransferse 1 (*Hprt*; HKG) FP (5' → 3'): AAGACAGCGGCAAGTTGAAT RP (5' → 3'): GGGCCTGTGTCTTGAGTTCA	NM_012583.2
(Rat) L1 (L1-3) FP (5' → 3'): GACCATCTGGAACCCTGGTG RP (5' → 3'): GGGCCTGTGTCTTGAGTTCA	DQ100473.1
(Rat) L1 (L1-Tot) FP (5' → 3'): GGAAGAGACCACCAACACTG RP (5' → 3'): GAAGGTTTAGCTCTCCCTCC	DQ100473.1 DQ100475.1 DQ100476.1 DQ100477.1 DQ100474.1 DQ100482.1

Legend: HKG, housekeeping gene; bp, base pairs; UTR, untranslated regions.

Table 2. Drug treatments for *in vitro* screening

Treatment	Mechanism of action	Concentration	Ref.
DMSO (control)	Vehicle for all drugs; served as control	0.1%	(1)
5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR)	Increase AMPK activity	1 mM	(1)
Caffeine (Caf)	Increase intracellular calcium release	5 mM	(1)
Rotenone (Rot)	Increase intracellular NADH levels	100 nM	(22)
Resveratrol (Res)	Increase SIRT activity	10 μM	(13)
Trichostatin A (TA)	Decrease HDAC activity	100 nM	(27)
5-Azacytidine (5-AZA)	Decrease DNMT activity	10 μM	(31)

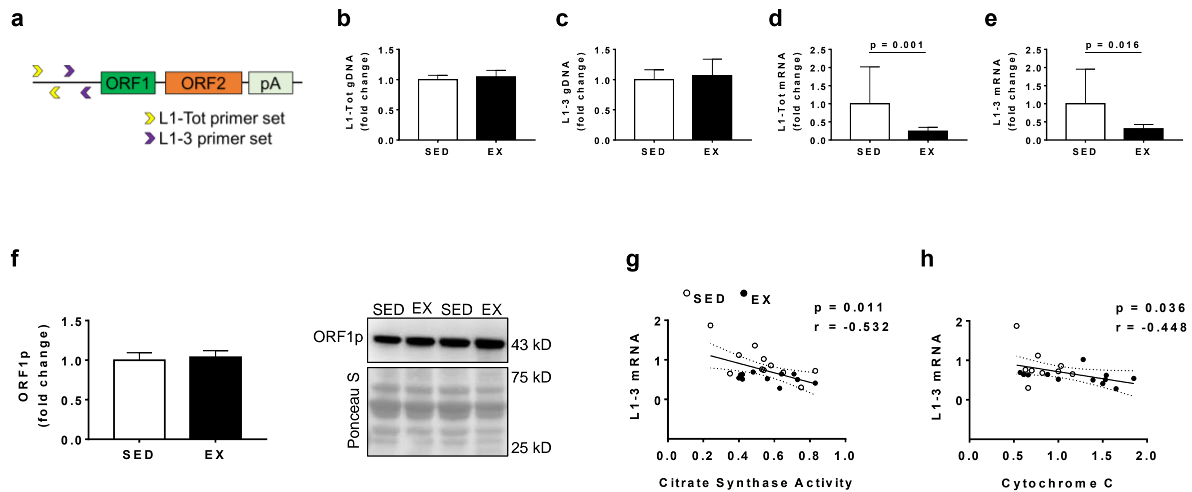

Figure 1. L1 primer design, skeletal muscle L1 DNA content, L1 mRNA expression, and correlations with select mitochondrial markers

Legend: Panel a depicts the general location of L1 primers. Panels b and c depict L1 DNA content differences between the groups using the L1-Tot and L1-3 primer sets described in the text. Panels d and e depict mRNA expression for L1-Tot and L1-3. Panel f depicts ORF1p protein expression with a Western blot representative image depicted next to figure. All data in panels b-f are presented as fold change relative to the SED group ±SD. Panels g and h represent associationed between L1-3 mRNa expression versus mitochondrial markers (CS activity and Cytochrome C, respectively).

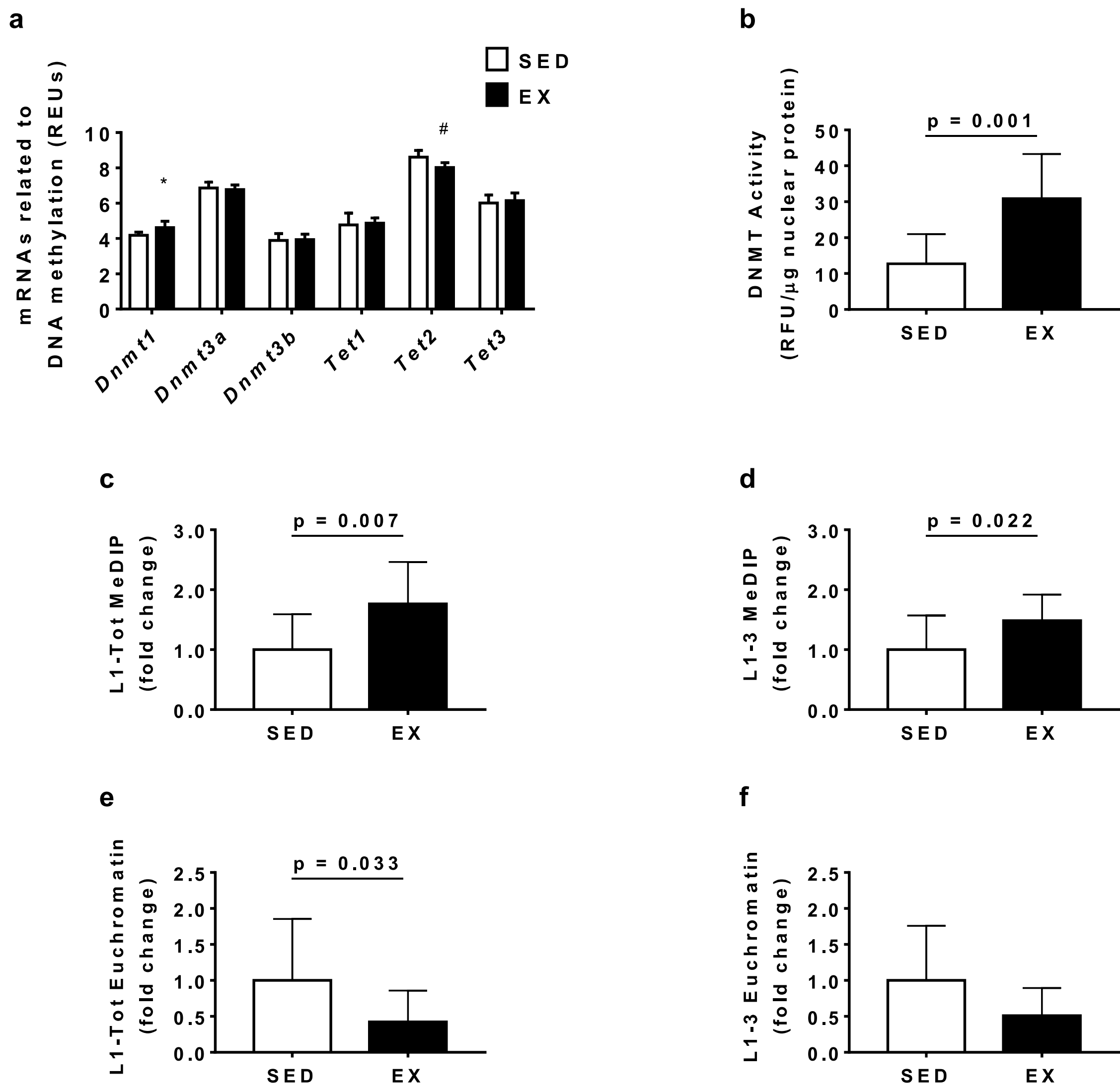

Figure 2. Skeletal muscle microarray results and L1 methylation changes

Legend: Panel a depicts microarray results for mRNAs of genes involved with DNA methylation between groups. Panel b depicts nuclear enzyme activity for DNMTs. Panels c and d depict methylated DNA immunoprecipitation (MeDIP) assay results for L1-Tot and L1-3. Panels e and f depict chromatin accessibility represented as amount of L1-Tot and L1-3 in euchromatin. For panel a, "*" signifies higher levels in EX versus SED ($p<0.05$), while "#" signifies lower levels in EX versus SED ($p<0.05$).

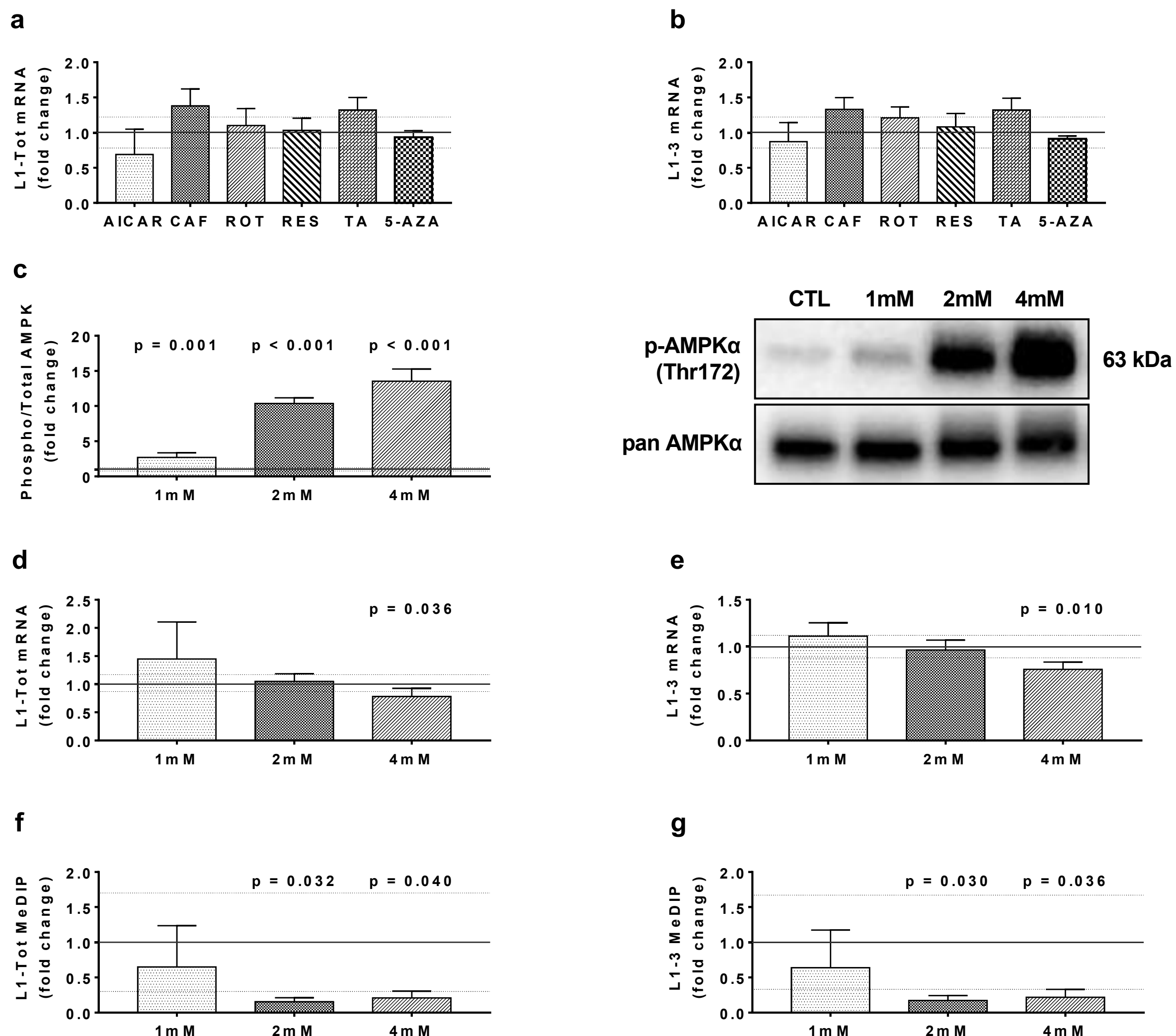

Figure 3. Cell culture experiments to determine possible exercise-specific pathways that regulate L1 mRNA expression in rat L6 myotubes

Legend: Panels a and b depict the mRNA expression for L1-Tot and L1-3 following 3-h treatments with a variety of drugs described in Table 2. Panel c depicts phosphorylation of AMPK (Thr-172) with increasing doses of AICAR alon with accompanying western blot representative image. Panels d and e show the effects of different AICAR doses on L1-Tot and L1-3 mRNA expression. Panels f and g show L1-Tot and L1-3 methylation measured via MeDIP. All statistical differences are relative to the DMSO control (DMSO presented as solid line at 1.0±SD in all figures). Abbreviations: CAF, caffeine; ROT, rotenone; RES, resveratrol;

TA, Trichostatin A; 5-AZA, 5-Azacytidine; 1mM, 2mM, and 4mM refer to the increasing concentrations of AICAR.

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