

**A New Perspective on Human Bipedalism: Using Functional Genomics as a
Window to Past Physiology**

by

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DISSERTATION ABSTRACT

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Doctor of Philosophy in Anthropology

Title: A New Perspective on Human Bipedalism: Using Functional Genomics as a Window to Past Physiology

Determining *when*, *why* and *how* the transition to bipedalism occurred is central to our field and critical to understanding the evolutionary history of traits that separate humans from other primates. This dissertation refines our understanding of hominin bipedal evolution by investigating the genetic basis of a trait not preserved in the fossil record that supports long-distance locomotion: fatigue-resistant slow myofiber-biased muscles. To address this, I first combined a meta-analysis with novel histochemical data from gorilla muscle to assess how distinct human muscle is compared to other mammals, and evaluated the effects of behavioral interventions (e.g., exercise) on human muscle myofiber composition. I found that humans are unique among African apes in having slow myofiber-biased muscles, but similar to sloths and slow lorises, suggesting that these species may have converged upon regulatory mechanisms shaping slow-biased muscles. Next, I used an evo-devo approach to characterize the epigenetic landscape of mouse muscles differing in slow myofiber content at embryonic (E18.5) and adult time points, identifying thousands of muscle-specific *cis*-regulatory elements (CREs) linked to myofiber differentiation and muscle physiology. I then tested if 12 candidate CREs were able to affect gene expression in a mouse skeletal muscle cell line, and found that all were functional, including nine enhancers (which increased gene expression) and three silencers (which decreased gene expression). Finally, I used evolutionary analyses to identify conserved muscle-specific CREs that harbor human-specific sequence changes potentially contributing to muscle endurance and bipedal efficiency. I identified 190 candidate CREs and tested if human- and chimpanzee-specific variants in six candidate CREs differentially affected gene expression in a human skeletal muscle cell line. While five candidates functioned as enhancers, species-specific variation within them did not lead to significant differences in gene expression. This dissertation situates ‘unique’ human musculature within a broader mammalian context, characterizes the genomic landscape of embryonic and adult mouse muscles differing in slow myofiber content, and provides a set of conserved, muscle-specific CREs relevant to human ($n = 190$) and primate ($n = 1,656$) evolution. As traits that “make us human” can also increase disease susceptibility, the CREs and pathways identified here may guide future interventions to reduce myofiber wasting, improving both the quality of life and lifespan.

This dissertation includes previously published co-authored material.

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DEDICATION

For my loved ones, and for those who have inspired me and continue to inspire others.

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1 INTRODUCTION

As biological anthropologists, we seek to understand the evolutionary origins of traits important to human (*Homo sapiens*) biology, particularly those that distinguish us from other primates. One of the most distinct characteristics of our species is our form of locomotion. Among primates, humans are unique in being habitual bipeds (i.e., exclusively walking on two limbs) and are the only extant species capable of endurance running¹. Whether walking or running, humans can locomote over long distances and for extended durations with remarkable efficiency. Quadrupedal chimpanzees (*Pan troglodytes*) cannot match the locomotor economy of human walking^{2,3}, and over marathon-length distances, humans can outrun almost all animals, including horses, especially in hot conditions⁴. This is particularly remarkable considering how recently this trait evolved in the human lineage compared to renowned cursorial athletes like dogs (*Canidae* ~10 million years⁵) and horses (*Equidae* ~55 million years⁶), whose running abilities were further refined over thousands of years of artificial selection⁷⁻⁹. In contrast, the human lineage diverged from quadrupedal apes only 7-8 million years ago (MYA)¹⁰, with the earliest evidence of long-distance bipedal locomotion appearing ~2 MYA in *Homo erectus*^{4,11,12}. Given this short evolutionary timescale, it is astonishing that some humans are now considered among the best long-distance, long-duration athletes on Earth^{1,13}. This raises a key question: what traits underlie this unique human ability, and were these traits under selection in the hominin (i.e., bipedal ape) lineage?

1.1 The evolution of hominin bipedal locomotion

Bipedalism is arguably the defining feature of hominin evolution, setting in motion a series of events (e.g., brain enlargement, language development, and technological innovation) leading to human expansion outside of Africa. Since the early nineteenth century, numerous theories have been proposed to explain *why* hominins became bipedal 4-7 MYA¹⁴⁻¹⁸, often citing changes to the environment and an increased reliance on hands for carrying resources (**Table 1.1**). Although considerable debate remains regarding the locomotor strategy of the last common ancestor (LCA) of humans and chimpanzees¹⁹ (i.e., orthograde arboreal suspension^{20,21}, terrestrial knuckle-walking^{22,23}, vertical climbing^{24,25}, arboreal bipedality²⁶, or generalized quadrupedalism^{27,28}) and which fossils provide the earliest definitive evidence of bipedalism²⁹, there is a broad consensus that australopiths were habitual bipeds by 4 MYA^{14,30}, and that *H. erectus* was capable of long-distance bipedal locomotion around 2 MYA¹¹ (**Fig. 1.1**). This view is supported by the discovery of more complete hominin specimens³¹⁻³⁸ and the widespread distribution of *H. erectus* across Africa and Eurasia³⁹. These specimens reveal key anatomical adaptations that facilitated this transition from earlier forms of locomotion to facultative (i.e., capable of walking on two legs), habitual, and, eventually, long-distance obligatory bipedalism.

Table 1.1. Summary of major hypotheses proposed to explain the evolutionary transition to terrestrial bipedalism.

Hypothesis	Summary	Citations
Savanna	Increasing aridity and seasonality 5-8 MYA reduced woody vegetation and gave rise to expansive woodland savannas. As tree cover declined and resources became more dispersed, arboreal apes were pressured to leave the trees and travel terrestrially to access safety and food. Bipedalism gave individuals a competitive edge over resource allocation and/or predator avoidance, ultimately enhancing their reproductive success.	40-47
Food Transport	In environments with sparse tree cover and intense competition for high quality resources (e.g., tubers, marrow, meat), bipedalism freed the arms for carrying. This allowed individuals to transport food from competitive foraging locations to private refuges to avoid sharing or theft. Those who could carry more food consumed more than their competitors, ultimately gaining a fitness advantage.	48-52
Tool/Weapon Transport	Bipedalism freed the arms for carrying tools and weapons, giving individuals a competitive advantage in acquiring high quality food resources and defending against predators. However, no widely accepted lithic technology has been definitively associated with early hominins like <i>Ardipithecus</i> or <i>Australopithecus</i> .	22,51,53,54
Vigilance	In more open savannas, smaller apes capable of adopting a bipedal stance, even intermittently, would be able to detect approaching predators earlier than non-bipedal apes, avoiding predation.	55
Provisioning	Bipedal males capable of carrying food more efficiently and exhibiting a tendency toward consistent provisioning would be highly desirable to females. Provisioning males would gain a fitness advantage through increased reproductive access, while females and their offspring would benefit from improved nutritional support.	56,57
Thermoregulation	In hot, open equatorial savannas, a bipedal posture would have significantly reduced exposure to solar radiation and thermal stress by minimizing surface area exposed to the sun and promoting heat dissipation. This would have allowed bipedal individuals to remain active throughout the day, increasing their overall food acquisition.	11,12,58-62
Agnostic Display	Recurrent violence between primate groups over resources often leads to injury and death. The peaceful, non-violent resolution of conflict through bipedal displays (e.g., bluffing or charging) would decrease morbidity and mortality in populations adopting this behavior and increase group fitness.	63,64
Small-Tree Postural Feeding	In woodland savannas, an upright, bipedal posture would have facilitated more efficient fruit harvesting by allowing individuals to reach higher branches and transport greater quantities of fruit more easily. This likely promoted bipedal movement between trees.	26,65-68

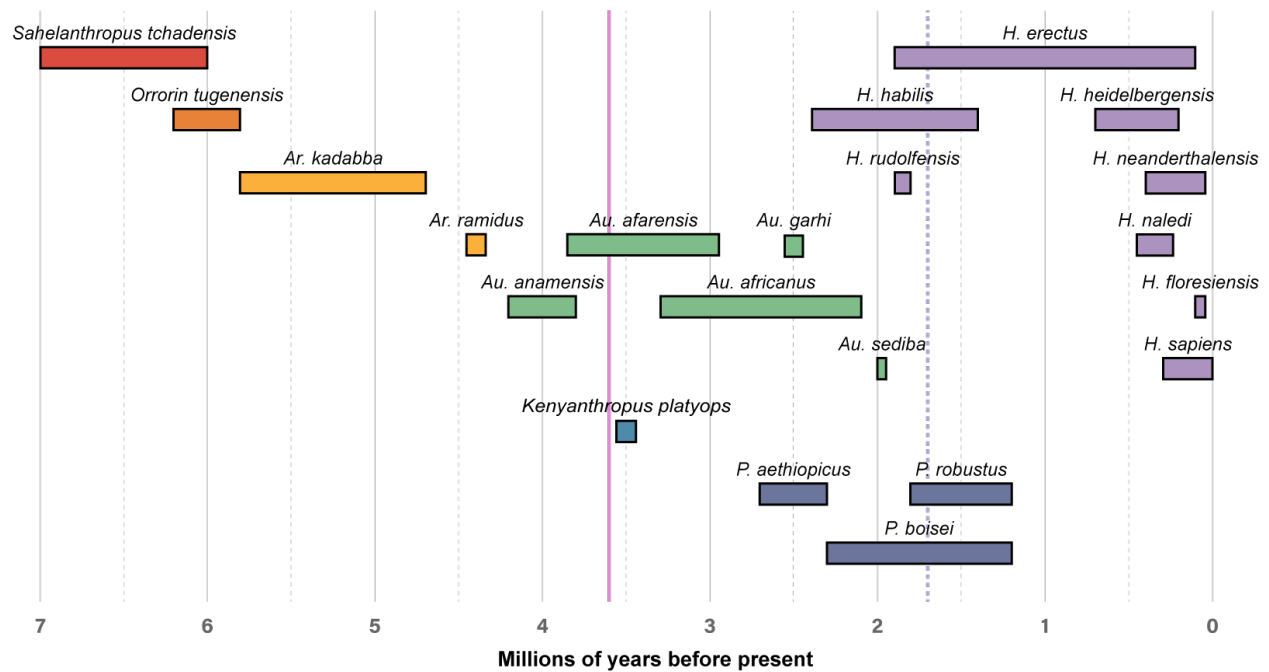


Fig. 1.1. Temporal distribution of proposed hominin taxa based on dates from the Smithsonian National Museum of Natural History. Informal groupings by color reflect taxonomic genus (red, *Sahelanthropus*; orange, *Orrorin*; yellow, *Ardipithecus*; green, *Australopithecus*; teal, *Kenyanthropus*; purple, *Paranthropus*; violet, *Homo*). No ancestor-descendant relationships are implied. The solid pink line marks the date of the Laetoli Footprints (3.6 MYA). The lavender dotted line marks the first fossil evidence of *H. erectus* out-of-Africa (1.7 MYA).

1.2 Adaptations for habitual bipedalism

Long-distance habitual bipedalism, and the traits facilitating it, did not evolve overnight, but rather emerged gradually through incremental changes to the hominin body that were refined over millions of years of natural selection. Although the fossil record is sparse and biased^{69,70}, it provides critical snapshots of hominin skeletal anatomy spanning the last 7 million years. These fossils enable researchers to trace the evolution of locomotor adaptations and perform comparative osteological analyses with living species to better understand early hominin behavior and locomotor performance. Through such investigations, a well-described suite of anatomical adaptations associated with bipedal locomotion have been identified (Fig. 1.2). These can be broadly characterized as adaptations for habitual bipedalism, and adaptations for long-distance, long duration (i.e., endurance) locomotion.

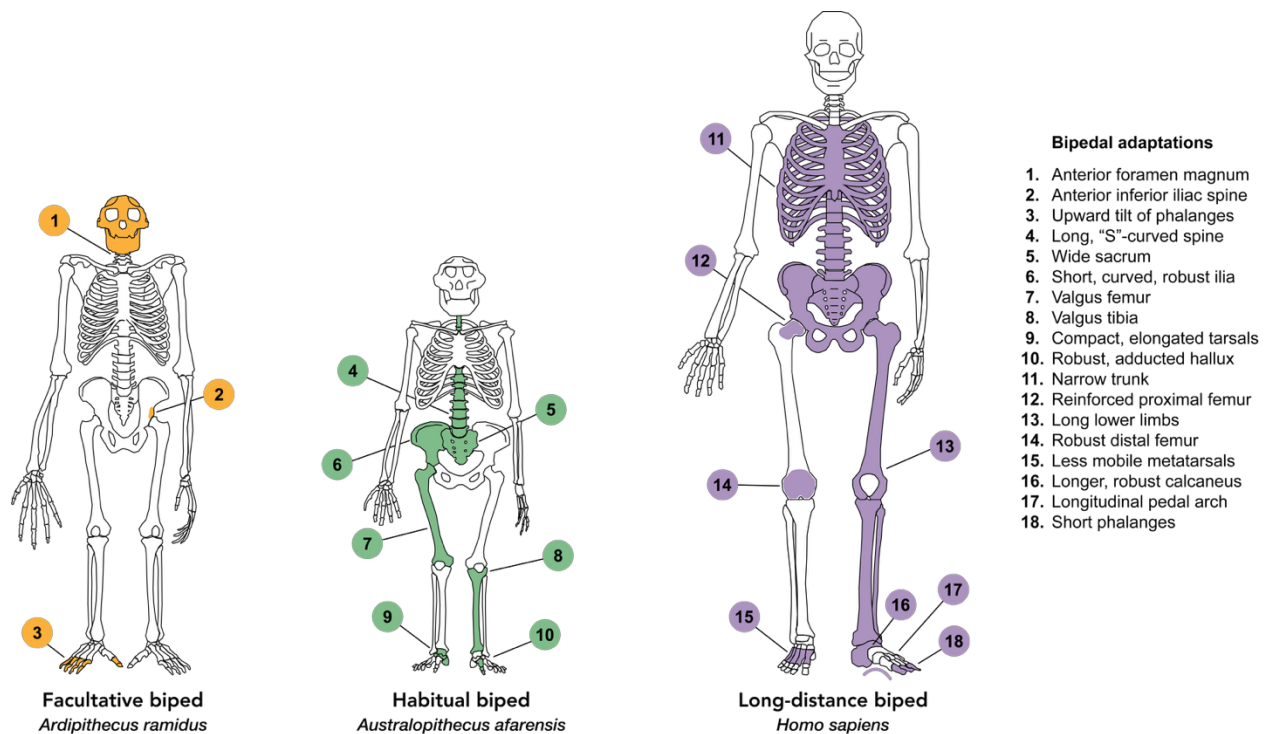


Fig. 1.2. Skeletal adaptations associated with facultative and habitual bipedalism as observed in the fossil record. Schematic illustrations show bipedal features present in a disputed early hominin (*Ardipithecus ramidus*), an early habitual biped (*Australopithecus afarensis*), and a long-distance biped (*H. sapiens*). Numbers correspond to the legend at right. Features are color-coded to match genus-level groupings in Fig. 1.1. Adapted from Lovejoy et al., 2009 and elucy.org.

One of the consequences of bipedal locomotion was an increased mechanical load on the lower limbs. Unlike quadrupeds, who distribute their body weight evenly across all four limbs, bipeds must support their weight on just two. To accommodate this, the hominin post-cranial skeleton underwent significant modifications. Many of these weight-bearing adaptations are especially apparent in the hominin foot. For example, hominin tarsals (ankle bones) became more compact to facilitate efficient weight transfer during toe-off and improve leverage during walking^{29,71-73}. Similarly, the midfoot became more compact, reducing joint mobility and providing a stable push off lever during bipedal walking^{29,71,72,74,75}. The hallux (big toe) also became more robust and less opposable (i.e., aligned in parallel with the other toes), functioning as a propulsive lever for upright walking rather than as a grasping digit for arboreal clambering^{29,72,76}.

The hominin spine also exhibits several derived features that support bipedal locomotion, including an elongated spinal column and a lumbar curve (lordosis). These adaptations enhance spinal mobility, absorb shockwaves transmitted to the skull during bipedal walking, compensate for an anteriorly-shifted obstetric load, improve the transfer of upper body weight to the hip joints, and reduce the effort of standing upright^{29,41,71,77,78}. While a more anteriorly positioned and horizontally oriented foramen magnum (the opening at the base of the skull where the spinal cord connects with the brain) is often interpreted as

evidence of a vertically oriented spine during locomotion^{29,41,71}, the reliability of this feature as a definitive marker of habitual bipedalism remains contested^{79–82}. Thus, the designation of fossil apes as hominins should not rely upon foramen magnum position alone.

At the base of the spine, the sacrum became wider, broadening the pelvis and allowing for greater pelvic rotation and a more efficient bipedal gait⁸³. The ilia (hip bones) became shorter and more curved, lowering the trunks' center of mass and bringing the center of gravity closer to the hip joints, enhancing balance and enabling lumbar lordosis^{17,72,77,83}. This curvature also expanded the attachment sites for hip-stabilizing muscles such as the gluteus maximus, which play a key role in maintaining the body's center of gravity during bipedal walking^{17,72,77,83}. Together, the widened sacrum and shorter, curved ilia formed the characteristic “bowl-shape” of the hominin pelvis, which better supports the increased load of an upright trunk^{29,41,71,72}. To accommodate this new pelvic morphology, the knees shifted medially relative to the hips, producing a valgus (adducted) angle that positions the knees more directly beneath the body's center of mass and reduces the effort of standing^{71,72,84,85}. Similarly, a valgus tibial position and more equally sized tibial condyles position the feet under the center of mass and enhance knee stability^{85–87}.

Together, these traits encompass a “bipedal suite” that evolved over millions of years and distinguish bipedal hominins from the quadrupedal apes. Interestingly, early bipeds like *Ardipithecus ramidus* (“Ardi”, ARA-VP-6/500) and *Au. afarensis* (“Lucy”, A.L. 288-1) possessed a mosaic of features adapted for both habitual bipedalism and arboreality^{14,18,27,57,88,89}. This duality highlights a key aspect of early hominin behavior: reliance on multiple modes of locomotion. The presence of derived bipedal adaptations (e.g., lumbar curve, bowl-shaped pelvis, less mobile midfoot, and valgus femur) indicates that early hominins were capable of walking terrestrially in open woodland savannas^{46,47}. Yet, the retention of primitive arboreal features (e.g., upwardly oriented shoulder joint, long arms, curved phalanges, and Ardi's opposable big toe) suggests that they remained heavily reliant on trees for survival. As a result, early hominins were not yet adapted for long-distance terrestrial ranging^{74,90}, and likely experienced reduced endurance, shortened daily travel distances, and contracted home ranges when they first adopted a bipedal locomotor strategy⁹¹. However, if the original utility of bipedalism was to traverse short distances, such as between trees in a woodland savanna, these limitations may not have posed a significant fitness disadvantage. Thus, this early, energetically inefficient form of bipedal locomotion was able to persist for millions of years, and was even utilized by members of genus *Homo*, including *Homo habilis*⁹² and the more recent *Homo naledi*⁹³.

1.3 Adaptations for endurance locomotion

H. erectus provides the first evidence of long-distance bipedal locomotion and is recognized as the first hominin to migrate outside of Africa, with fossil evidence appearing in Georgia by 1.7 MYA,

Indonesia by 1.5 MYA and China by 1.2 MYA^{39,94}. Their broad geographic dispersal is attributed in part to their human-like body proportions and to endurance-enhancing features also present in *H. sapiens*⁹⁴. Because long-distance walking and running impose a greater degree of mechanical and thermoregulatory stress on the body than a short jaunt between the trees, many adaptations are hypothesized to have evolved in response to these challenges (**Fig. 1.1**). To withstand an increased mechanical load, the femoral head and distal femur enlarged, helping the hip and knee joints bear greater amounts of stress during leg extension and improving the leverage of the hip abductors (muscles of the lateral thigh)^{29,71,72,83}. The femoral neck also shortened and became asymmetrically reinforced with cortical bone and a dense lattice of trabeculae (fine, porous bone), enhancing resistance to the bending stresses associated with bipedal locomotion⁷². Similarly, the calcaneus (heel bone) became more robust, helping it to dissipate peak compressive forces and absorb energy during heel-strike under an increased bipedal load^{71,95,96}. An enlarged calcaneus also facilitated the creation of a stable longitudinal pedal arch by expanding ligament attachment sites running from the arch of the foot to the tibia¹. This spring-like structure enhances shock absorption and elastic energy storage during foot strike while maintaining midfoot rigidity for powered plantar flexion during toe off^{29,71,72,74,75,97,98}. In addition to improving the efficiency of bipedal walking and standing⁹⁹, the longitudinal arch protects against lower limb injury^{100,101}.

Like other cursorial species, both *H. erectus* and *H. sapiens* exhibit significantly elongated lower limbs (legs) and more compact feet (i.e., short toes)¹. These traits increase stride length and optimum walking speeds while limiting foot rotation, thereby reducing the energetic cost of walking and running^{3,83,91,102–104}. Additionally, lower and wider shoulders, shorter forearms and a narrower trunk (thorax, waist and pelvis) enhance trunk counter-rotation, increasing the counter-balancing forces generated by arm-swinging¹.

Still, many traits that support long-distance bipedal locomotion are not preserved in the fossil record, making their evolutionary origins difficult to trace. These soft tissue adaptations include an increased density of eccrine sweat glands¹⁰⁵, a reduction in body hair⁵⁹, an elongated Achilles tendon¹⁰⁶, an enlarged gluteus maximus proprius¹⁰⁷, shorter myofiber lengths¹⁰⁸, mouth breathing¹, and the presence of a nuchal ligament^{1,109}. Of particular interest for this dissertation is the high proportion of fatigue-resistant slow myofibers in human lower limb muscles, which enhance muscle endurance¹⁰⁸.

1.4 Myofibers and movement

The human body is composed of over 600 skeletal muscles, which account for roughly 55% of individual body mass^{110,111} and support a broad range of vital functions (i.e., breathing¹¹², the generation of heat¹¹³, amino acid storage¹¹⁴, glucose homeostasis¹¹⁵, mechanical support¹¹⁶, and joint stability¹¹⁷) but

most notably locomotion¹¹⁸ and body posture¹¹¹. Each skeletal muscle consists of long, cylindrical muscle cells known as myofibers, which contain chains of specialized, rod-like organelles called myofibrils¹¹⁹. Myofibrils are composed of repeating arrays of actin and myosin filaments that are organized into individual contractile units called sarcomeres¹²⁰ (**Fig. 1.3**). Muscle contraction occurs through interactions between actin and myosin filaments within each sarcomere. In this process, the energy molecule adenosine triphosphate (ATP) binds to the myosin head and causes it to detach from actin. ATP is then broken down into adenosine diphosphate (ADP) and phosphate, releasing energy and facilitating the repositioning of the myosin head. The energized myosin then reattaches to actin and pulls the actin filament toward the sarcomere's center (the M-line), shortening the H-zone and producing contraction. When many sarcomeres contract simultaneously, the myofibrils shorten, leading to contraction of the myofiber and ultimately the entire muscle¹²⁰. Variations in sarcomere length¹²⁰, and in the proportion and type of contractile proteins like actin¹²¹, titin¹²², troponin¹²³, tropomyosin¹²⁴, myosin light chain (MyLC)¹²⁵, and myosin heavy chain (MyHC) isoforms^{126–129} can significantly affect muscle performance by altering muscle contraction speeds, force output, and resistance to fatigue.

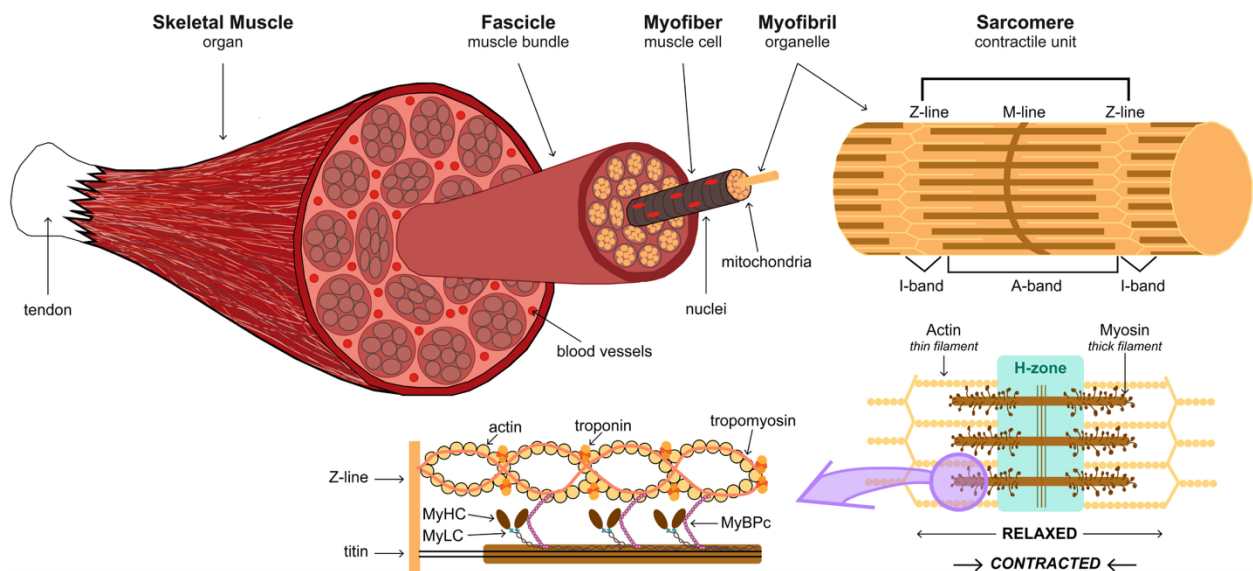


Fig. 1.3. Structural organization of skeletal muscle. Schematic illustrating the hierarchical arrangement from the whole muscle organ to the muscle cell (myofiber), with expanded detail showing interactions between actin (thin filament) and myosin (thick filament) that underlie sarcomere contraction.

Individual myofibers can be categorized into distinct types based on their unique physiological properties^{130–132}, when they arise during myogenesis¹³³, and contractile protein composition¹³⁴. Because myofiber function (e.g., contraction time, strength, and resistance to fatigue) largely depends on MyHC isoform expression^{128,129}, MyHC isoforms (e.g., slow-oxidative MyHC- β , fast-oxidative-glycolytic

MyHC-IIa, fast-intermediate MyHC-IIx, fast-glycolytic MyHC-IIb) are commonly used as molecular markers of myofiber type. While myofibers can be heterogenous (e.g., they can co-express pairs of MyHC isoforms)^{135–137}, adult myofibers can be broadly separated into two distinct types: slow myofibers, which predominantly express slow MyHC- β , and fast myofibers, which predominantly express one of the fast MyHC-II isoforms¹³³.

Slow myofibers are endurance myofibers, characterized by their resistance to fatigue and ability to recover after prolonged activity. Due to slower motor domain kinetics, slow myofibers hydrolyze ATP at a slower rate than fast myofibers, and, thanks to their high density of mitochondria, can replenish ATP lost during hydrolysis via aerobic oxidative phosphorylation^{131,138–141}. This efficient use and regeneration of cellular energy allow slow myofibers to maintain contractile activity for prolonged periods of time^{127,131,142}. In addition, slow myofibers exhibit several cellular features that promote continued responsiveness during sustained contraction, including a more active Na⁺/K⁺ pump, a low synaptic depression (i.e., the depletion of neurotransmitter-containing synaptic vesicles after neuronal stimulation) and lower mean Ca²⁺ uptake by the sarcoplasmic reticulum^{127,131,142}. Blood vessels in muscles enriched with slow myofibers are also more prone to vasodilation, allowing for increased oxygen delivery during physical activity and further enhancing their endurance capacity^{7,131}.

In contrast, fast myofibers generate velocity and power (force x velocity) several-fold greater than slow myofibers. Their high rate of ATP hydrolysis and tendency to rely on glycolysis to synthesize ATP create a metabolic environment rich in inorganic phosphate and protons, making them more susceptible to fatigue^{143–146}. Fast myofibers also exhibit several cellular features that support rapid contractile responses to phasic innervation, including a higher Na⁺ channel density, faster Ca²⁺ uptake, a larger sarcoplasmic reticulum volume, and an elevated abundance of Ca²⁺ transport proteins (e.g., calsequestrin)^{127,131,142}. However, the rapid influx of signaling ions (e.g., Ca²⁺, K, Na) during phasic innervation leads to swift ATP depletion and a rapid decline in responsiveness, ultimately limiting the duration of fast myofiber contractile activity and contributing to their tendency to fatigue more rapidly¹³¹.

1.5 Muscle myofiber content and locomotor strategy

Due to the varied functional demands of different muscles, they can vary in the proportions of fast and slow myofibers (i.e., muscle myofiber composition)^{111,147}. Muscles with a high proportion of fast myofibers (e.g., extensor digitorum longus) are typically involved in propulsion and dynamic stabilization, whereas muscles with a high proportion of slow myofibers (e.g., soleus) tend to be used in postural or endurance-based tasks^{148,149}. Shifts in myofiber composition can significantly affect contractile and metabolic properties at both the myofibril and whole-muscle level^{150,151}, profoundly impacting organism movement. For example, experimentally altering the physiological properties of muscle tissue

has been shown to significantly improve muscle endurance and increase average distance travelled per day in mice¹⁵², highlighting the importance of myofiber content in shaping organism movement and locomotor specialization.

Decades of fiber-typing research has revealed that muscle myofiber content varies considerably across species and is shaped, in part, by locomotor strategy (**Fig. 1.4**). Given the fundamental role of muscle tissue in movement and the unique physiological properties of each myofiber type, it is likely that muscle myofiber composition has been a repeated target of selection across diverse mammalian lineages to better support the way that each species moves through its environment. For example, the muscles of small-bodied mammals such as bats^{153–155} and mice^{156–158} are dominated by fast myofibers, which support high-powered, short-duration behaviors like flying and sprinting. These species also express an extremely fast-contracting MyHC isoform (MyHC-IIb) that is absent from the limb muscles of larger mammals, suggesting that body size in addition to locomotor strategy plays a role in shaping muscle myofiber composition^{142,159–162}. Similarly, the forelimbs of fossorial species like armadillos¹⁶³ and moles¹⁶⁴ exhibit a higher proportion of fast myofibers, which support rapid digging. Vertical leapers like marmosets, whose mean power output during jumping is twice that of humans, display a remarkably high proportion (>99%) of fast myofibers in lower limb extensors like the vastus lateralis, compared to just 51–58% in humans^{165–167}. In contrast, orangutans, whose arboreal lifestyle requires slow, sustained movements, exhibit a greater proportion of slow myofibers in the lower limb than chimpanzees, whose terrestrial movements involve quicker, more powerful plantar flexion¹⁶⁷.

Humans differ considerably from other primates in their high proportion of fatigue-resistant slow myofibers in lower limb musculature. Differences in muscle myofiber content are especially apparent between humans and their closest living relatives (chimpanzees and bonobos) and contribute to species-specific differences in muscle performance. Although chimpanzees outperform humans in pulling and jumping tasks¹⁰⁸, they are unable to match the locomotor economy of human walking². This is attributed to the predominance of power-generating fast myofibers (~67%) in chimpanzee musculature^{108,167}, and the predominance of fatigue-resistant slow myofibers (~67%) in humans^{157,165,166,168–171} (**Table 1.2**). These findings suggest that slow-biased lower limb muscles are an adaptation for endurance walking, running, and bipedal postural support, and that selection for more efficient bipedal locomotion shaped hominin muscle physiology over the past 7–8 million years^{13,108,131}. However, because muscles are not typically preserved in the fossil record, it remains unclear *when* slow myofiber-biased muscles first appeared and how hominin muscle physiology changed over time. Thus, an alternative to the fossil record is to reconstruct the evolutionary history of this trait using genomics to determine whether slow-biased muscles were under selection in the hominin lineage.

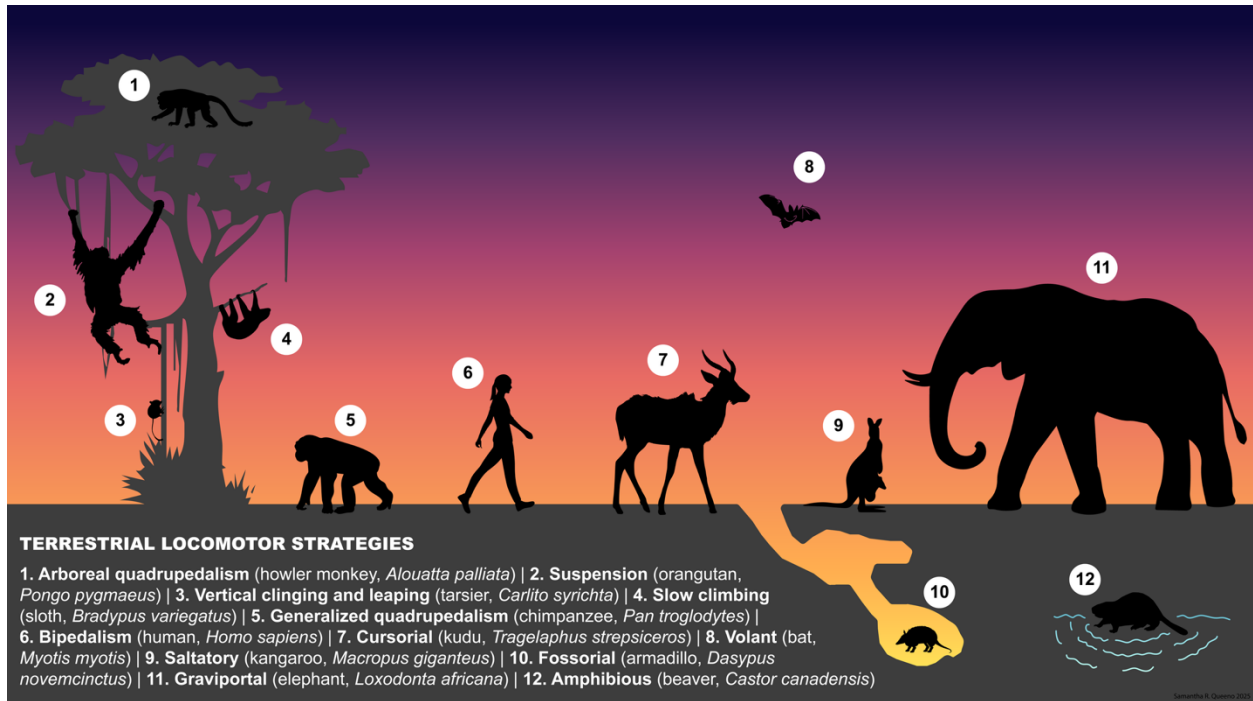


Fig. 1.4. Diverse terrestrial locomotor strategies in mammals. Schematic illustrating major terrestrial locomotor strategies employed by extant mammalian species. Numbered silhouettes correspond to the legend below. Silhouettes are illustrative and may not be to scale.

Table 1.2. Slow myofiber content (%) in human and chimpanzee lower limb muscles.

Muscle	Anatomical Location	Human ($n = 6$) ¹⁶⁴	Chimpanzee ($n = 3$) ¹⁰⁸
Adductor brevis	Thigh	69.4	31.9
Adductor longus	Thigh	61.7	42.3
Adductor magnus	Thigh	76.1	43.2
Biceps femoris	Thigh	75.9	34.2
Gracilis	Thigh	65.5	20.2
Pectineus	Thigh	63.2	21.8
Rectus femoris	Thigh	49.7	39.8
Sartorius	Thigh	67.4	25.8
Semimembranosus	Thigh	78.6	39.0
Semitendinosus	Thigh	60.5	25.1
Vastus intermedius	Thigh	63.0	51.9
Vastus lateralis	Thigh	48.3	34.4
Vastus medialis	Thigh	52.8	33.8
Extensor digitorum longus	Leg	54.9	38.6
Extensor hallucis longus	Leg	83.0	18.3

Muscle	Anatomical Location	Human ($n = 6$) ¹⁶⁴	Chimpanzee ($n = 3$) ¹⁰⁸
Gastrocnemius	Leg	61.7	18.6
Peroneus brevis	Leg	61.9	33.9
Peroneus longus	Leg	55.6	38.3
Popliteus	Leg	89.1	26.1
Soleus	Leg	68.1	52.4
Tibialis anterior	Leg	79.4	32.8
Tibialis posterior	Leg	80.0	38.9
Gemellus inferior	Pelvic/Gluteal	74.5	10.6
Gemellus superior	Pelvic/Gluteal	78.9	47.5
Gluteus medius (average)	Pelvic/Gluteal	86.0	25.1
Iliacus	Pelvic/Gluteal	76.7	44.2
Obturator externus	Pelvic/Gluteal	68.9	28.7
Obturator internus	Pelvic/Gluteal	67.3	28.1
Piriformis	Pelvic/Gluteal	84.3	31.8
Quadratus femoris	Pelvic/Gluteal	92.5	42.6

1.6 Using DNA to study the evolution of a trait unobservable in the fossil record

Given the high degree of protein-coding (gene) sequence similarity between humans and chimpanzees, phenotypic differences between these two species are largely attributed to species-specific changes within evolutionarily conserved non-coding regions of the genome¹⁷². These regions often act as spatiotemporal regulators of gene expression (e.g., *cis*-regulatory elements, CREs, such as enhancers that *enhance* gene activity and silencers that *silence* gene activity) that influence *where*, *when*, and *how much* a gene is expressed over the course of development¹⁷³. Mutations within CREs can alter the timing or location of gene expression at critical developmental stages, leading to phenotypic variation and evolutionary divergence between species^{174–184}. However, because individual genes can affect multiple, seemingly unrelated traits (pleiotropy), gene expression is spatiotemporally regulated by discrete *trans*-acting factors that limit, and localize, the effects they have (**Fig. 1.5**). Thus, a CRE mutation may only alter gene expression at a specific time point or tissue (e.g., embryonic soleus), allowing for slight modifications in gene regulatory networks without disrupting them entirely¹⁸⁵. Such modifications are thought to be a major source of evolutionary novelty¹⁸⁶, and have already been shown to impact primate evolution and the emergence of human-specific traits^{187–194}.

In this way, human-specific mutations within CREs that regulate the expression of genes involved in myofiber development and physiology may be critical to understanding the uniqueness of human skeletal

muscle and the evolution of endurance locomotion in the hominin lineage. However, apart from MyHC “super-enhancers”^{195,196}, the genetic mechanisms controlling muscle myofiber composition and fatigue-resistance remain poorly defined, and the impact of selection on these regions is largely unknown. Identifying these mechanisms, particularly in regions under accelerated evolution in humans compared to other species, will allow us to functionally validate the effects of human- and chimpanzee-specific mutations on gene expression. For a more detailed discussion, see Chapters 2, 4 and 5.

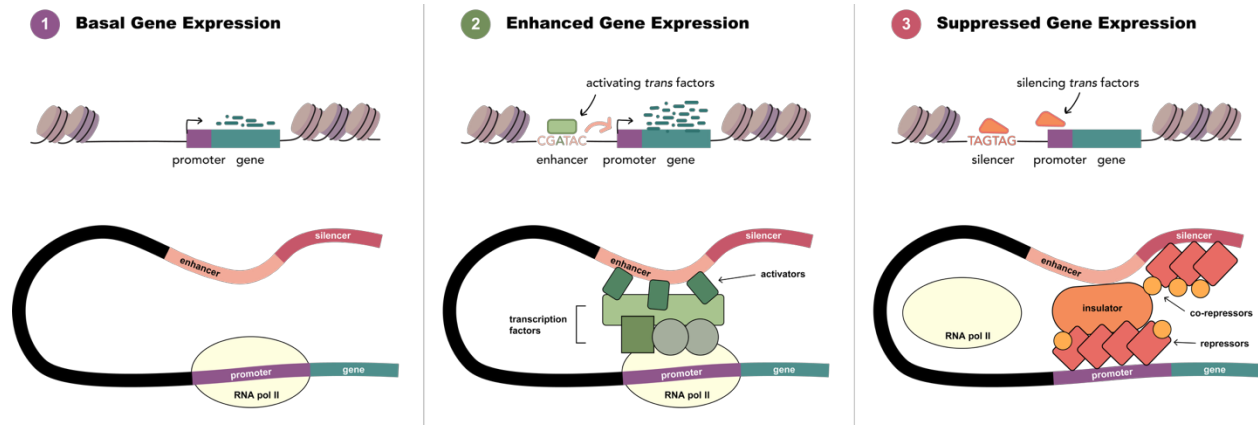


Fig. 1.5. *Cis*- and *trans*-acting components of gene regulation. Schematic illustrating how *trans*-acting factors can bind to distal *cis*-regulatory elements (CREs), such as enhancers or silencers, to modulate gene transcription by RNA polymerase. Panel 1 shows basal transcription (gene expression) driven solely by the core promoter. Panel 2 shows enhanced transcription facilitated by transcription factor binding that brings an enhancer near the promoter, stabilizing RNA polymerase at the transcription start site. Panel 3 shows transcriptional suppression mediated by repressor proteins that bind to the promoter or silencer elements, thereby preventing RNA polymerase recruitment.

1.7 Overview of the dissertation

The overall objective of this dissertation is to identify evolutionarily conserved muscle-specific CREs located in regions of human-specific accelerated evolution associated with skeletal muscle endurance, and to determine whether species-specific variation within these CREs leads to differences in gene expression *in vitro*. The central hypothesis is that selection for more efficient bipedal locomotion drove the fixation of human-specific substitutions within conserved, muscle-specific CREs, resulting in a higher proportion of fatigue-resistant slow myofibers in the human lower limb. This dissertation is organized into four primary data chapters.

Chapter 2: Human and African ape myosin heavy chain content and the evolution of hominin skeletal muscle. Co-authored by Peter J. Reiser, Caley M. Orr, Terence D. Capellini, Kirstin N. Sterner, Matthew C. O'Neill and myself, this chapter investigates the evolutionary basis of differences in muscle myofiber composition between humans and their closest living relatives, the African apes. Specifically,

we ask whether the high proportion of slow myofibers in human lower limb musculature is simply a byproduct of larger body size or whether it reflects selection acting on a trait that enhances long-distance bipedal locomotion. To address this, we combine a comprehensive meta-analysis with novel histochemical data generated from gorilla skeletal muscle to assess how distinct human muscles are compared to a diverse range of mammalian species. We also evaluate whether behavioral interventions, such as exercise, can significantly shift human muscle toward a chimpanzee-like, fast myofiber-biased phenotype.

Chapter 3: Meta-analysis data of skeletal muscle slow fiber content across mammalian species. Co-authored by Kirstin N. Sterner, Matthew C. O'Neill, and myself, this chapter expands on the meta-analysis introduced in Chapter 2. We analyze trends in the data as they relate to sampling bias, technological advancement, muscle type, body size, taxonomy, and locomotor strategy. Mammalian skeletal muscle slow myofiber composition data was collected from 269 eligible studies, encompassing more than 200 skeletal muscles across 174 mammalian species, and made publicly accessible via Mendeley Data. This dataset provides a valuable resource for those interested in muscle physiology and the evolution mammalian phenotypic diversity.

Chapter 4: *Cis*-regulatory elements shape myogenesis and myofiber differentiation in late embryonic and adult mouse muscles. Co-authored by Alexander S. Okamoto, Damien M. Callahan, Matthew C. O'Neill, Terence D. Capellini, Kirstin N. Sterner and myself, this chapter investigates how gene regulation shapes muscle development in a mouse model and whether differences in gene regulation between myofiber types can be detected during late embryogenesis (E18.5). Using an evolutionary developmental biology framework, we characterized patterns of gene expression (via RNA-seq¹⁹⁷) and chromatin accessibility (via ATAC-seq^{198,199}) in mouse hind limb muscles that differ in slow myofiber content at both a late embryonic (E18.5) and adult time point. This analysis identified key genes and pathways that are differentially regulated across time points and muscle types, as well as thousands of candidate CREs associated with muscle identity and myofiber type. Twelve candidate CREs were functionally validated *in vitro* using a mouse skeletal muscle cell line (C2C12), with nine acting as enhancers and three as silencers.

Chapter 5: Identifying potential *cis*-regulatory elements that may shape human muscle evolution. Using the mouse muscle epigenetic datasets generated by Alexander S. Okamoto for Chapter 4, this chapter investigated whether evolutionarily conserved mammalian CREs have contributed to human skeletal muscle endurance and long-distance bipedal evolution. Building on the candidate CREs identified in Chapter 4, we focus on muscle-specific CREs shared between humans and mice that overlap regions of human-specific accelerated evolution, which may have been co-opted to enhance slow myofiber content in humans. We tested the effect of human- and chimpanzee-specific variation within six CRE candidates

on gene expression using a human skeletal muscle cell line. While nine species-specific candidates acted as enhancers *in vitro*, none exhibited significant species-specific differences in gene expression, though notable trends were observed. This work generated a set of muscle-specific CREs of potential evolutionary importance to humans ($n = 190$) and primates ($n = 1,656$), establishing a foundation for future functional studies.

Chapter 6 synthesizes the results of the preceding chapters (2-5) and discusses implications for future research.

2 HUMAN AND AFRICAN APE MYOSIN HEAVY CHAIN CONTENT AND THE EVOLUTION OF HOMININ SKELETAL MUSCLE

From Queeno, S. R., Reiser, P. J., Orr, C. M., Capellini, T. D., Sterner, K. N., & O'Neill, M. C. (2023). Human and African ape myosin heavy chain content and the evolution of hominin skeletal muscle. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 281, 111415.

2.1 Introduction

Humans are unique among African apes (i.e., chimpanzees and gorillas) and other primates in the size and shape of our lower back, pelvis, and hind/lower limbs. Our suite of musculoskeletal traits interact so as to enhance our bipedal locomotor capabilities as compared to living African apes^{2,200}, facilitating long-distance, overground travel and other long duration, muscle-driven activities. While skeletal traits are important determinants of locomotor performance and are under selection in the earliest hominins^{18,66}, skeletal muscle tissue also plays a fundamental role in the mechanics and energetics of movement. Yet, its contribution to hominin locomotor evolution is still not well understood. This is due to the limitations of the hominin fossil record and the near absence of comparative studies of human skeletal muscle biology, beyond individual-muscle mass and architectural measurements^{201,202}.

Measurements of pulling^{203–206} and jumping²⁰⁷ tasks in humans and chimpanzees indicate that chimpanzee skeletal muscle can outperform human skeletal muscle on a mass-specific basis, by about 1.5 times on average¹⁰⁸. While direct measurements of the contractile characteristics of single muscle fibers (i.e., maximum isometric forces, maximum shortening velocities) indicate that humans and chimpanzees are quite similar, these two species differ substantially in their distribution of myosin heavy chain (MyHC) isoforms throughout their skeletal muscle¹⁰⁸. Indeed, while human skeletal muscles possess a predominance of MyHC I (i.e., slow fibers), the same muscles in chimpanzees possess about half as much, instead exhibiting a predominance of MyHC II (i.e., IIa, IIc; fast fibers). This difference is relevant to locomotor performance capabilities, as MyHC isoform content affects whole-muscle contractile mechanics in both *in vitro* experiments^{131,208} and Hill-based muscle model simulations of maximum dynamic force and power^{108,209}. In particular, the MyHC isoform content of a muscle can influence its rate of contraction in response to a neural signal, as well as the shape of its force-velocity relationship²⁰⁸. The metabolic cost of isometric force production and fatigability varies among muscle fibers of different MyHC content, with whole muscles that exhibit a predominance of slow fibers having the lowest costs and highest fatigue resistance²¹⁰. As such, the predominance of MyHC I content within human skeletal muscle may reflect a shift within the hominin lineage, affecting both the mechanics and energetics of

locomotor performance.

Still, it remains unclear whether the predominance of MyHC II or fast fibers is a characteristic of the pelvis and hind limb muscles of African apes as a group or simply a characteristic of chimpanzees alone. Direct measurements of skeletal muscle MyHC content of a gorilla species would help address this and further contextualize human patterns within the larger ape and primate clades. Gorilla skeletal muscle measurements also provide an important assessment of the size independence of human and African ape MyHC content. Whereas chimpanzee body masses are smaller than humans on average, both Western and Eastern gorillas can exceed human mean body mass by more than two times²¹¹. As such, commonalities among chimpanzees and gorillas would suggest that any human-African ape MyHC isoform composition differential is likely untethered from the increase in hominin body mass over the past 7 to 8 million years (Ma)^{212,213}, which likely began with a common ancestor of the *Pan* and *Homo* lineages that was similar in size to chimpanzees^{214,215}.

Understanding how humans and African apes compare to other terrestrial mammals across a wide body mass range is also needed to provide broader comparative context to human MyHC I content. Since 1973, the skeletal muscle fiber composition (defined based on mATPase, immunohistochemistry, MyHC content, RNA expression, etc.) of several nonhuman primate species have been studied (**Appendix A**), but only macaques²¹⁶, galagos^{217,218}, slow lorises^{217,218}, and mouse lemurs²¹⁹ have representative sampling across their pelvis and hind limbs (i.e., $n \geq 8$ muscles). An initial assessment of these and other studies indicated that the percent MyHC I or slow fiber content increases with larger body mass in terrestrial mammals, albeit with considerable interspecific variation (**Appendix A**)¹⁰⁸. However, a more comprehensive sampling of the currently available literature across the terrestrial mammalian size range will further clarify the extent to which humans or African apes depart from this size-scaling pattern and help identify what other terrestrial mammals are slow fiber dominant.

Of course, skeletal muscle fiber composition is expected to be responsive to age and some behavioral interventions, although the magnitude of this effect is difficult to determine from one or two studies alone. Human muscle fiber composition has been measured in the pelvis and lower limb muscles of both young (22 yrs)¹⁶⁵ and older adults (83 yrs)¹⁶⁶ and has been subject to a broad range of behavioral interventions, from immobilization to increased exercise. While muscle mass is exceptionally responsive to these factors^{220,221} the responsiveness of muscle fiber composition may be quite modest by comparison. A recent meta-analysis of decreased activity levels (e.g., bed rest, leg unloading, detraining) on human lower limb muscle found a trivial 1-2% decrease in MyHC I content, on average ($n = 451$ subjects)²²². It is possible that increased activity levels due to exercise or strength training may have a more substantial effect on MyHC I content, but there is no comparable survey of the existing human experimental data. Such data would provide greater insight into the extent to which the human-African ape differential in

MyHC isoform composition is attributable to behavioral shifts in life or may be better understood as an evolvable musculoskeletal trait under selection between species.

Here, we combine new MyHC isoform content data from pelvis and hind limb muscles of a Western gorilla (*Gorilla gorilla*) with existing chimpanzee (*Pan troglodytes*) data, as well as comprehensive data collation and meta-analysis of skeletal muscle fiber composition studies from 1965 to 2022 to better understand the distinctiveness of human skeletal muscle MyHC I content. Based on these results, we suggest that the substantial MyHC I content in human pelvis and lower limb skeletal muscle may be best understood as an evolvable musculoskeletal trait under selection in the hominin lineage, perhaps for reducing muscle metabolism and enhancing fatigue resistance to aerobic muscle contractile behavior. We hypothesize that molecular mechanisms that underlie muscle fiber composition are potential targets for selection during hominin evolution. Given the importance of MyHC gene regulation during muscle fiber development^{133,223,224}, it is likely that species-specific differences in adult MyHC content, such as those observed between humans and African apes, are due to species-specific changes in regions of the genome that regulate the expression of MyHC genes and their MyHC isoforms. As such, we highlight potential targets of selection in the genome (e.g., enhancer regions, a specific type of regulatory element) that may play an important role in hominin skeletal muscle evolution.

2.2 Materials and methods

2.2.1 *Gorilla gorilla* muscle MyHC content

The MyHC isoform distribution of *Gorilla gorilla* was determined for samples (10-20 mg) of 35 pelvis and hind limb muscles from one adult, female gorilla. The animal had been living in a zoo and died of natural causes prior to muscle sampling. After death, the cadaveric remains were kept fresh frozen at -20 °C until dissection. The pelvis and hind limb were free of visible pathology. Prior to dissection, the specimen was CT scanned using a Philips-Gemini TF64. Serial scans of each bone were collected at a slice thickness of 0.67 mm with 0.35 overlap (voltage: 120kV; current 320 mA). The CT scanner output a stack of DICOM image files that were imported, rendered, and measured in Mimics Research 19.0 (Materialise n.v., 2016). Femoral head superior-inferior diameter (FHSI) predicted an adult body mass of 85.3 kg using a gorilla-specific reference sample, although an African ape reference sample prediction was similar (FHSI: 41.33 mm; African Ape Eqn: 83.7 kg)²²⁵.

To determine MyHC isoform composition, the skin and fascia were removed around the pelvis and hind limb, then individual muscles were identified. A 2 cm x 0.5 cm x 0.5 cm muscle tissue sample was taken from two locations of each muscle in the same manner as a previous study¹⁰⁸. The MyHC isoform expressed in each sample was identified using SDS-PAGE. The composition, preparation, staining and

densitometric scanning of the gels were identical to those described previously¹⁰⁸. The amount of each MyHC isoform in the sample that expressed multiple isoforms was calculated as a percentage of the total amount of MyHC. The three MyHC isoforms that were detected on protein gels were identified based on commonalities with previous human and chimpanzee gels¹⁰⁸.

The distribution of MyHC isoforms within 35 pelvis and hind limb muscles of the gorilla was compared using an analysis of variance (ANOVA), with Tukey HSD post-hoc pairwise comparisons between MyHC isoforms. Two-sample t-tests were used to conduct pairwise comparisons between the average fraction of MyHC I in the gorilla mean and the individual-subject means for the chimpanzee and two human datasets. For interspecific comparisons, the mean MyHC I content across all pelvis and hind/lower limb muscles was calculated for each individual, then the between-individual mean and standard deviation were determined. This reflects the variance in mean MyHC I content between individuals studied in each species. Two human datasets were used, representing both young (22 yrs; $n = 14$ muscles)¹⁶⁵ and old adults (83 yrs; $n = 37$ muscles)¹⁶⁶, and permitting an initial assessment of age effects on mean MyHC I content across the pelvis and lower limb.

2.2.2 *Terrestrial mammal muscle fiber composition comparisons*

2.2.2.1 *Eligibility criteria and study selection*

Published, peer-reviewed data were compiled for meta-analysis between June 1 2021 and November 30 2022 following a structure similar to Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA)²²⁶, facilitating transparency and ease of reproducibility. Terms relating to mammalian skeletal muscle fiber composition (red, Type I, β , MHC I, MyHC I, slow twitch, fatigue resistant, slow oxidative, slow fiber, slow myosin isozyme) were queried using academic search systems (Google Scholar, PubMed, and JSTOR) and library databases for relevant primary articles. Reference lists in relevant articles were thoroughly investigated for eligible studies. No restrictions were set on the date of publication.

Eligible studies were published in the English language and provided muscle fiber composition data from at least one skeletal muscle from a species belonging to class Mammalia. Any data collected after a behavioral intervention (e.g., % fiber content after a treadmill running regime) were excluded.

2.2.2.2 *Data extraction*

The following data were recorded from the text, tables, figures, and supplementary materials of included studies when available: sampled species' common name, scientific name, sex, age, method for classification of muscle fiber composition, slow muscle fiber terminology used (e.g., MyHC I, MHC I, Type I, β , slow oxidative, red or slow twitch), number of individuals sampled, average body mass (kg),

muscle(s) sampled and average percent slow fiber content (% fibers).

In most studies, body mass was reported; for those studies that did not report a body mass the species mean was taken from published references^{227,228}. When muscle fiber content was recorded as the percentage of fast muscle fibers within a skeletal muscle, the proportion of slow muscle fibers was derived as 100 minus the total proportion of fast muscle fibers. If slow muscle fiber content was reported from multiple sampling sites across a single muscle (e.g., superficial and deep), the average across the sampling sites was recorded as the percent slow muscle fiber content of that muscle.

Given that most studies were published between 1965 and 1999, binomial and common names were updated when necessary to reflect the current understanding of phylogenetic relationships. Taxonomic data (i.e., class, infraclass, magnorder, superorder, order, suborder, infraorder, parvorder, clade, superfamily, family, subfamily, tribe, and subspecies) provided by NCBI Taxonomy²²⁹ was recorded for each species when available.

For interspecific comparisons a minimum sampling threshold of 8 pelvis and hind/lower limb muscles per species was considered representative. Studies meeting the minimum sampling threshold were used for two analyses: (i) one comparing the average slow fiber content across all measured pelvis and hind/lower limb muscles per species and (ii) one comparing the mean slow fiber content across a set of 8 matched pelvis and hind/lower limb muscles per species, which controlled for individual muscle variation. The matched muscle condition included muscle composition measurements from the *mm. gluteus medius*, *biceps femoris*, *semimembranosus*, *semitendinosus*, *vastus lateralis*, *extensor digitorum longus*, *gastrocnemius*, and *tibialis anterior*. This subset of muscles represented the three main anatomical regions (i.e., pelvis, thigh, leg) while permitting the greatest number of species to be included. When multiple studies existed for the same species, slow fiber content was averaged by muscle across studies, and the average of recorded body masses was used as the pooled species average body mass. This both increases the number of animals sampled and permits the inclusion of data from multiple labs and techniques, which should reflect measurement reproducibility.

2.2.2.3 Interspecific scaling analyses

To incorporate the potential statistical non-independence of species due to shared common ancestry, the scaling relationship of muscle slow fiber content with $\log_{10}$ body mass was evaluated using phylogenetic generalized least squares (PGLS). The phylogenetic structure of the analyzed species was taken from published mammalian trees²³⁰. The PGLS parameters were estimated using the Comparative Analyses of Phylogenetics and Evolution in R package²³¹ with the parameter lambda (λ) fixed at 0 (star phylogeny) and 1 (Brownian motion) and empirically estimated using a maximum-likelihood (ML) approach²³². The three different model fits (i.e., 0, 1, ML) were compared using a likelihood ratio test. Human, chimpanzee and gorilla MyHC I content z-scores were calculated as $z = (x_i - \mu_i)/\sigma$, where x_i is the

average fraction of MyHC I of species i , μ_i is the i species mean predicted from the regression fit, and σ is the standard deviation of the sample. To detect regression fit outliers, phylogenetic residuals were studentized by dividing the residual by the square root of their variance. Studentized residuals greater than an absolute value of 3 were considered outliers and removed²³³. Phylogenetic residuals were also standardized by dividing the residual by the square root of the expected value.

2.2.3 Human intervention muscle fiber content comparisons

2.2.3.1 Eligibility criteria and study selection

Humans are the best-studied species in terms of the response of muscle fiber content to behavioral interventions and are of clear, direct relevance for hominin locomotor evolution. As such, published, peer-reviewed data were compiled for meta-analysis between June 1 and November 30 2022, following a structure similar to PRISMA²²⁶. Terms relating to human exercise (human exercise, human running, human training, strength training, endurance training, sprint training, detraining, cycling) and skeletal muscle fiber composition (red, Type 1, β , MHC I, MyHC I, slow twitch, fatigue resistant, slow oxidative, slow fiber, slow myosin isozyme) were used to query academic search systems (Google Scholar, PubMed, JSTOR) and library databases for relevant primary articles. Reference lists in relevant articles were thoroughly investigated for eligible studies. No restrictions were set on the date of publication.

Eligible studies were published in the English language and provided muscle fiber composition data from human lower limb muscles both before and after a behavioral intervention. This permitted a change in mean slow fiber content (%) over time to be calculated. As such, only longitudinal studies were included; all cross-sectional studies were excluded from consideration. Interventions that included a countermeasure (e.g., bed rest with exercise or bed rest with nutritional countermeasure²³⁴) were also excluded, although the mean effects of these countermeasures all fall within the range determined here. No criteria were set on participant sex as no significant sex-based differences in skeletal muscle response to training have been identified^{235,236}.

2.2.3.2 Data extraction

The following data were recorded from the text, tables, figures, and supplementary materials of included studies when available: intervention type, intervention duration (hours, days, weeks, months), type(s) of exercise participants were subjected to, skeletal muscle sampled, number of participants, participant sex, average participant mass (kg), average participant age (years), average pre-intervention skeletal muscle slow fiber content (% fiber), average post-intervention skeletal muscle slow fiber content (% fiber), standard deviation, and method for classification of muscle fiber composition. Study results were then categorized into one of nine distinct behavioral interventions based on the activity under

investigation: bed rest, immobilization, environmental, spaceflight, endurance training, strength training, sprint training, dynamic training, and detraining. See definitions of each intervention in **Table 2.3**.

When muscle fiber content was recorded as the percentage of fast muscle fibers within a skeletal muscle, the proportion of slow muscle fibers was derived as 100 minus the total proportion of fast muscle fibers. If multiple intervention time points were reported along a time series, only slow fiber content at the first and last time points were recorded.

2.2.3.3 Statistical analyses

The delta of each study was calculated as the mean difference in slow fiber content at the start and end of each unique behavioral intervention for each studied lower limb muscle. For each of the nine intervention categories, a pooled intervention effect size was calculated across studies. This was done as a weighted average based on sample size, such that studies with a larger number of subjects had a larger weight on the intervention mean. The weighted intervention mean across all lower limb muscles and the weighted intervention mean of *m. vastus lateralis* alone were calculated. A negative delta indicated a decrease in slow fiber content, while a positive delta indicated an increase in slow fiber content.

The weighted intervention means were then compared to the mean difference in slow fiber content between humans and African apes (gorillas and chimpanzees) for all studied pelvis and hind/lower limb muscles, as well as for the *m. vastus lateralis* alone. For these muscles the human average was taken from Tirrell et al.¹⁶⁶ and Johnson et al.¹⁶⁵ and compared to that of chimpanzees¹⁰⁸ and the gorilla (this study) to determine the degree of difference between humans and each African ape.

2.3 Results

2.3.1 Gorilla MyHC isoform content is similar to chimpanzees

The distribution of MyHC isoforms within 35 pelvis and hind limb muscles of the gorilla indicate a predominance of MyHC II fibers, with a significant bias towards MyHC IId, on average ($F_{(2,114)} = 11.55$, $p = 0.000027$; **Fig. 2.1a**; **Table 2.1**). The MyHC IId content was significantly higher than MyHC I ($p = 0.00022$) or MyHC IIa ($p = 0.00015$). There are significant differences between African apes and humans (H) in mean MyHC I content across the pelvis and hind/lower limb ($F_{(3,122)} = 96.93$, $p < 0.0001$). Specifically, chimpanzees (c) and gorillas (G) were similar ($p = 0.6702$), while both species differed from young (G v. H: $p = 0.00012$; C v. H: $p = 0.00075$) and old adult humans (G v. H: $p = 0.00011$; C v. H: $p = 0.00012$) (**Fig. 2.1b**). The difference between the old and young adult humans was not significant ($p = 0.147$).

Muscle-matching across species to the young human muscle count ($n = 14$ muscles)¹⁶⁵ results in similar species-mean differentials (G: $29.7 \pm 8.1\%$; C: $33.4 \pm 11.9\%$; H: $61.8 \pm 14.6\%$, $55.8 \pm 5.7\%$) as in the

full, unmatched accounting. Similarly, all three species exhibit a similar pattern of African ape-human differentiation across the three regions within the pelvis and hind/lower limb (pelvis, thigh, leg) (**Fig. 2.1c**). Among the two human datasets, the older adults exhibit a larger fraction of MyHC I isoforms in their pelvis and thigh muscles than the younger adults, while the leg musculature is similar between groups.

Fig. 2.1 (next page). MyHC isoform distributions of gorilla, chimpanzee and human skeletal muscles. (a) Gorillas exhibit a MyHC II dominant distribution among the three MyHC isoforms across 35 skeletal muscles. *P* value is from an ANOVA. (b) Humans exhibit a significant bias towards slow MyHC I fibers in their skeletal muscle with measurements ranging from the between-subjects mean $\pm$ s.d. of (i) $69.0 \pm 13.7\%$ ($n = 37$ muscles, Tirrell et al.¹⁶⁶; T) to (ii) $55.6 \pm 5.7\%$ ($n = 14$ muscles, Johnson et al.¹⁶⁵; J). This differentiates humans (T: $n = 6$; J: $n = 6$) from the between-subject mean $\pm$ s.d. of $31.5 \pm 3.3\%$ in chimpanzees ($n = 3$) and 30.8% in the gorilla ($n = 1$). (c) The predominance of slow MyHC I fibers in human skeletal muscle is distinct from African apes for all major anatomical regions, including the pelvis, thigh and leg.

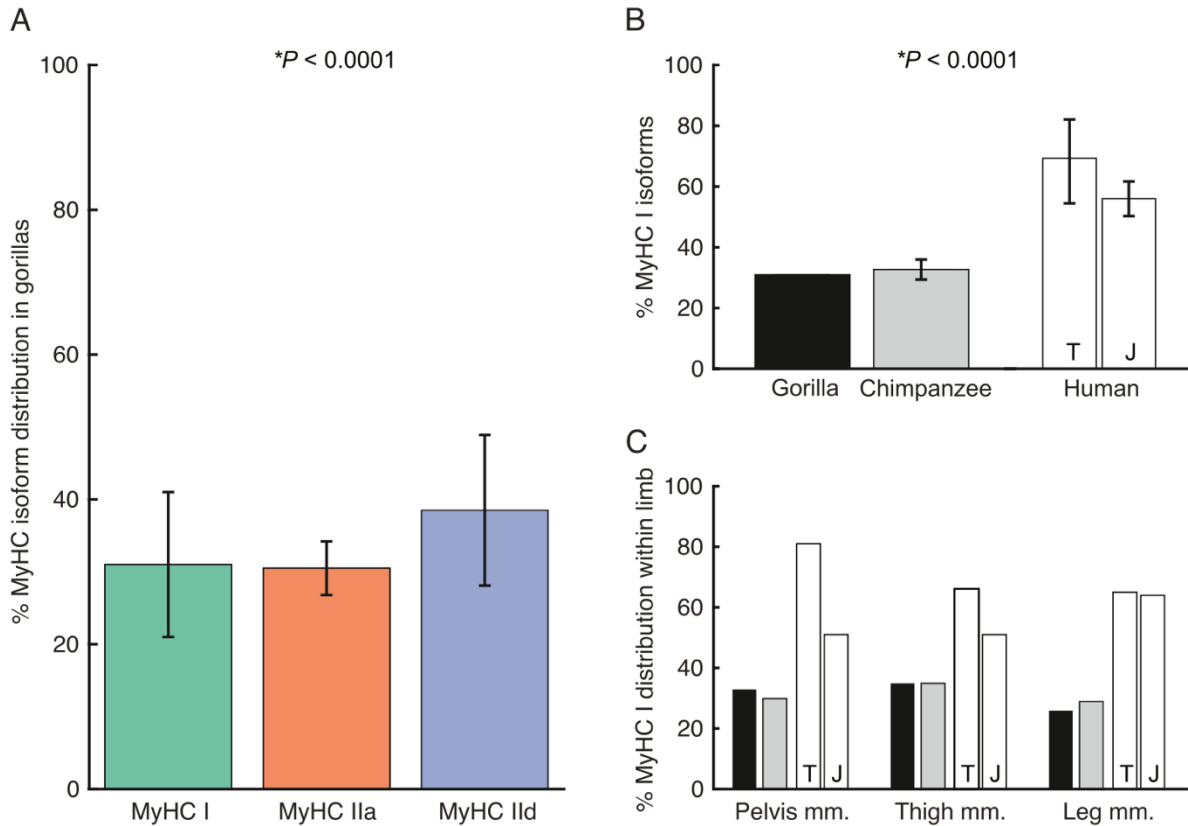


Table 2.1. The mean myosin heavy chain (MHC) isoform content of 35 pelvis and hind limb muscles of *Gorilla gorilla* ($n = 1$).

Muscle	MHC I (%)	MHC IIa (%)	MHC IIc (%)
Adductor brevis	26.9	31.9	41.3
Adductor longus	45.6	41.4	13.1
Adductor magnus			
Adductor magnus ischiocondylaris	53.5	25.8	20.8
Adductor magnus pubofemoralis	34.7	37.6	27.8
Biceps femoris			
Biceps femoris, long head	40.9	25.2	34.0
Biceps femoris, short head	26.7	28.5	44.9
Extensor digitorum longus	26.1	28.8	45.3
Extensor hallucis longus	31.4	26.2	42.6
Flexor digitorum fibularis	15.5	27.2	57.4
Flexor digitorum tibialis	17.7	27.1	55.2
Gastrocnemius lateralis	20.2	35.2	44.7
Gastrocnemius medialis	22.1	31.6	46.3
Gemellus inferior	17.2	32.4	50.5
Gemellus superior	27.4	30.1	42.7
Gluteus maximus			
Gluteus maximus ischiofemoralis	39.4	22.9	37.7
Gluteus maximus proprius	27.2	33.0	39.9
Gluteus medius	33.0	32.0	35.1
Gluteus minimus	29.5	30.2	40.4

Muscle	MHC I (%)	MHC IIa (%)	MHC IIb (%)
Gracilis	24.8	32.5	42.7
Iliopsoas			
Iliacus	33.9	30.7	35.4
Psoas major	20.6	29.5	49.9
Obturator externus	29.9	30.1	40.1
Obturator internus	23.2	27.0	49.8
Pectineus	30.1	31.9	38.0
Peroneus (fibularis) brevis	26.7	33.6	39.8
Peroneus (fibularis) longus	21.3	32.9	46.0
Piriformis	39.4	34.1	26.6
Quadratus femoris	50.9	29.2	20.0
Rectus femoris	28.4	30.1	41.5
Sartorius	26.3	34.4	39.4
Semimembranosus	38.4	26.6	35.2
Semitendinosus	26.7	31.4	41.9
Soleus	45.7	25.9	28.5
Tensor fascia lata	26.2	33.0	40.9
Tibialis anterior	21.2	34.5	44.5
Tibialis posterior	32.3	30.6	37.2
Vastus intermedius	55.3	33.8	11.0
Vastus lateralis	28.3	27.1	44.7
Vastus medialis	36.8	28.2	35.0
Mean	31.0	30.5	38.5
Standard deviation	10.0	3.7	10.4

2.3.2 *Humans are distinct from African apes and most other terrestrial mammals in muscle slow fiber content*

A total of 385 terrestrial mammal muscle fiber composition studies were identified. Of these, 115 were excluded because: (i) muscle fiber composition data were not provided ($n = 76$), (ii) muscle fiber composition data provided were ambiguous ($n = 15$), (iii) muscle fiber composition data were provided for an unidentified muscle ($n = 2$), (iv) muscle fiber composition data were provided for cardiac fibers ($n = 2$), (v) muscle fiber composition data were provided for single muscle fibers ($n = 4$), (vi) muscle fiber composition data were provided for a mammal under experimental conditions ($n = 4$), (vii) the study duplicated another dataset ($n = 2$), (viii) the source was secondary ($n = 7$), or (ix) the source was a published abstract ($n = 3$). The remaining 270 studies were published between 1965 and 2022. Skeletal muscle fiber composition data were collated for 175 species spanning 15 mammalian orders (see

Appendix A).

Of these, 35 species across 12 mammalian orders met the minimum sampling threshold of 8 hind/lower limb muscles (**Table 2.2**), ranging in size from a Dsinezumi shrew (0.0094 kg) to an African savanna elephant (1,542 kg). The total number of pelvis and hind/lower limb muscles sampled was 8 to 37 muscles per species, with humans and African apes being the best represented species. Twelve of the

studies meeting the minimum sampling threshold included data for the 8 matched pelvis and hind/lower limb muscles (i.e., *mm. gluteus medius, biceps femoris, semimembranosus, semitendinosus, vastus lateralis, extensor digitorum longus, gastrocnemius, tibialis anterior*). As such, the reduced, muscle-matched sample included 21 species, spanning the same size range.

PGLS scaling analyses revealed that greater $\log_{10}$ body mass predicted higher hind/lower limb slow fiber content across both the larger, unmatched (coefficient $\pm$ s.e. 7.51 ± 2.36 , $\lambda = 0.74$, 95% CI [0.12, 0.94], $r^2 = 0.21$, $t_{1,33} = 3.18$, $p = 0.003$) (**Fig. 2.2a**) and smaller muscle-matched (estimate $\pm$ s.e. 10.83 ± 3.61 , $\lambda = 0.54$, 95% CI [NA, 0.91], $r^2 = 0.29$, $t_{1,19} = 3.00$, $p = 0.007$) (**Fig. 2.2b**) datasets. In both cases, the ML model fit was significantly different from a star phylogeny in the unmatched, but not the matched sample ($\lambda = 0$; $p = 0.0461$ and $p = 0.1253$, respectively) and from Brownian motion in both samples ($\lambda = 1$; $p = 0.0001$ and $p = 0.0008$). Humans exhibit a positive z-score relative to this size-scaling relationship (2.23), whereas African apes exhibit negative z-scores (C: -0.66; G: -1.08). This pattern is similar for both the unmatched and muscle-matched datasets.

Only four terrestrial mammal species are slow fiber dominant (i.e., > 50% slow fibers) in their pelvis and hind/lower limb muscles (i.e., humans, slow loris and two sloths) in the unmatched interspecific sample. However, none of these species – including humans – were determined to be outliers with respect to the studentized residuals of the size-scaling relationship. In the reduced, muscle-matched sample, humans are the only primate that are slow fiber dominant, with the substantial human-African ape differential being maintained (**Fig. 2.2b**).

Fig. 2.2. (next page). Size scaling of slow fiber distribution across body size. (a) The relationship between $\log_{10}$ body mass and average percent slow fiber content from all available pelvis and hind/lower limb muscles ($n = 35$ species). (b) The relationship between $\log_{10}$ body mass and average percent slow fiber content from eight matched pelvis and hind/lower limb muscles (*mm. gluteus medius, biceps femoris, semimembranosus, semitendinosus, vastus lateralis, extensor digitorum longus, gastrocnemius, tibialis anterior*) ($n = 21$ species). Dotted line represents a slow fiber composition of 50%, above which denotes species that are slow fiber dominant. The solid line represents the slope and intercept of the PGLS regression of $\log_{10}$ body mass on slow fiber content. Data points are colored by taxonomic order. Species belonging to order Primates are distinguished with icons.

Table 2.2. Studies meeting the minimum threshold ($n \geq 8$ hind/lower limb muscles) for use in interspecific size scaling analysis of terrestrial mammals.

Order	Common name	Scientific name	No. of subjects	Avg. body mass (kg)*	No. of muscles	Avg. muscle slow fiber content (%)	Reference
Artiodactyla	Cattle, Japanese black	<i>Bos taurus</i>	3	680*	8	30.6	237
	Corriedale sheep	<i>Ovis aries</i>	6	45*	12	35.9	238
	Corriedale sheep	<i>Ovis aries</i>	7	58	14	34.1	238
	Pig, De-Kalb strain	<i>Sus scrofa domesticus</i>	5	107.5	15	28.5	239
Carnivora	Dog	<i>Canis lupus familiaris</i>	3	25.3	31	44.5	240
	Cheetah	<i>Acinonyx jubatus</i>	2	36	23	22.7	241
	Cat	<i>Felis catus</i>	1	3*	27	22.4	218
	Cat	<i>Felis catus</i>	3	3*	14	32.1	242
Didelphimorphia	Virginia opossum	<i>Didelphis virginiana</i>	7	2.488*	10	43.3	243
Diprotodontia	Brush-tailed bettong	<i>Bettongia penicillata ogilbyi</i>	1	1.018	8	20.0	244
Eulipotyphla	Japanese water shrew	<i>Chimarrogale platycephalus</i>	1	0.0385	16	0.0	164
	Dsinezumi shrew	<i>Crocidura dsinezumi</i>	1	0.0094	16	0.0	164
	Asian house shrew	<i>Suncus murinus</i>	2	0.1005	18	0.0	164
	Asian house shrew	<i>Suncus murinus</i>	5	0.04083	13	0.0	245
	Japanese shrew mole	<i>Urotrichus talpoides</i>	2	0.0162	16	0.0	164
	Small Japanese mole	<i>Mogera imaizumii</i>	1	0.065	16	0.0	164
	Japanese mole	<i>Mogera wogura</i>	2	0.111	16	0.0	164
Lagomorpha	New Zealand White rabbit	<i>Oryctolagus cuniculus domesticus</i>	1	2.65	8	13.0	246
Perissodactyla	Horse	<i>Equus ferus caballus</i>	6	482	13	24.6	247
Pilosa	Brown-throated sloth	<i>Bradypus variegatus</i>	5	3.74	12	89.2	248
	Hoffmann's two-toed sloth	<i>Choloepus hoffmanni</i>	4	4.875	12	62.8	248
Primates	Crab-eating macaque	<i>Macaca fascicularis</i>	3	4.6	9	40.8	216
	Gray mouse lemur	<i>Microcebus murinus</i>	12	0.0645	15	18.7	219
	Senegal bushbaby	<i>Galago senegalensis</i>	1	0.275*	21	15.3	218
	Senegal bushbaby	<i>Galago senegalensis</i>	7	0.275*	8	22.3	249

Order	Common name	Scientific name	No. of subjects	Avg. body mass (kg)*	No. of muscles	Avg. muscle slow fiber content (%)	Reference
	Senegal bushbaby	<i>Galago senegalensis</i>	7	0.1715	30	18.1	217
	Western gorilla	<i>Gorilla gorilla</i>	1	85.3	35	30.3	THIS STUDY
	Human	<i>Homo sapiens</i>	6	78.5	14	55.3	165
	Human	<i>Homo sapiens</i>	6	61.3	37	68.8	166
	Chimpanzee	<i>Pan troglodytes</i>	3	26.5	35	31.2	108
	Sunda slow loris	<i>Nycticebus coucang</i>	1	1.16*	15	56.7	218
	Sunda slow loris	<i>Nycticebus coucang</i>	3	1.16*	30	56.1	217
Proboscidea	African elephant	<i>Loxodonta africana</i>	1	1542*	14	47.6	250
Rodentia	Cape dune mole-rat	<i>Bathyergus suillus</i>	5	2	19	33.4	251
	Natal mole-rat	<i>Cryptomys hottentotus natalensis</i>	6	0.097	20	27.6	251
	Cape mole-rat	<i>Georychus capensis</i>	6	0.18	20	21.4	251
	Guinea pig	<i>Cavia porcellus</i>	1	0.629	22	17.4	218
	Syrian hamster	<i>Mesocricetus auratus</i>	3	0.146	19	14.3	252
	Small five-toed jerboa	<i>Allactaga elater</i>	2	0.12	9	29.3	253
	Swiss Webster mouse	<i>Mus musculus</i>	12	0.0246	26	6.4	156
	Sprague Dawley rat	<i>Rattus norvegicus domestica</i>	1	0.17*	25	13.2	218
	Sprague Dawley rat	<i>Rattus norvegicus domestica</i>	6	0.4614	23	16.2	254
	Sprague Dawley rat	<i>Rattus norvegicus domestica</i>	3	0.429	28	19.2	255
	Sprague Dawley rat	<i>Rattus norvegicus domestica</i>	6	0.323	27	8.6	256
Scandentia	Common treeshrew	<i>Tupaia glis</i>	8	0.123	30	17.8	217

*Sample mean body mass was not provided; therefore, mass is the mean for the species

2.3.3 Human behavioral interventions have a small impact on muscle slow fiber content

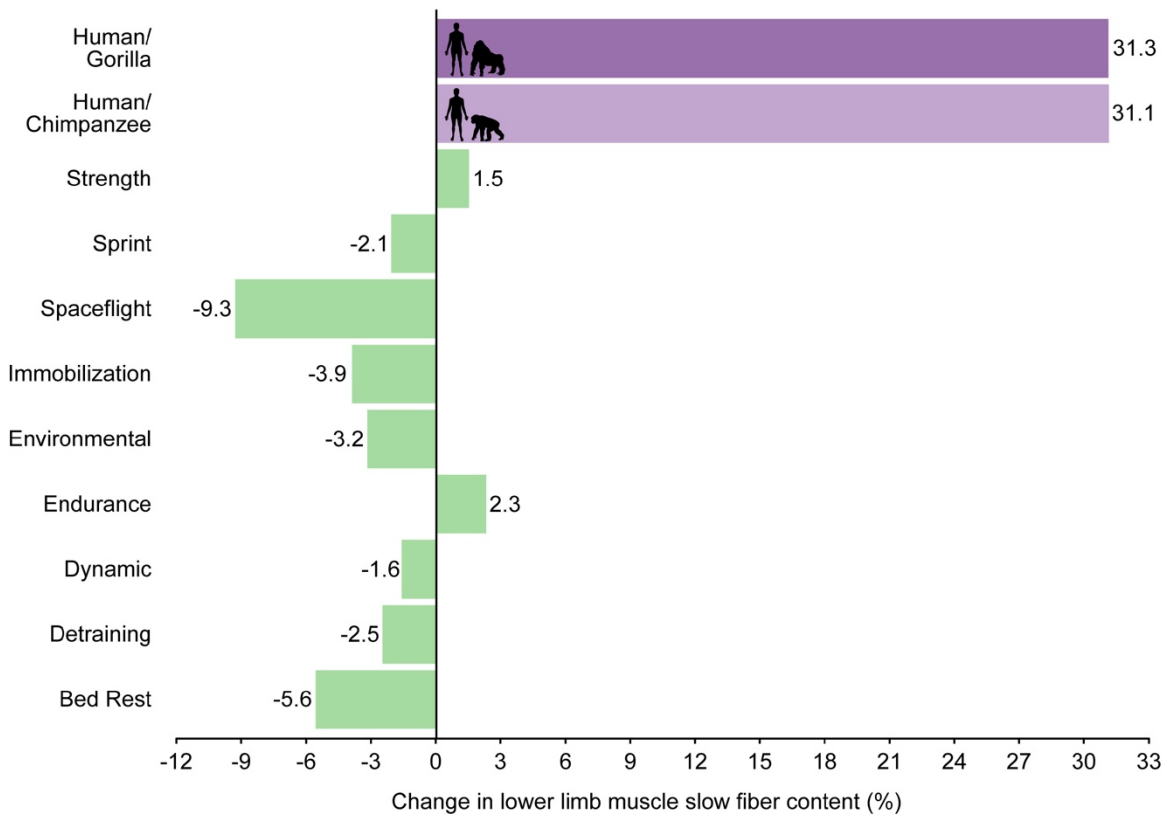
A total of 256 human intervention studies were identified. Of these, 131 studies were excluded because: (i) muscle fiber composition data were not provided ($n = 28$), (ii) pre- or post-intervention muscle fiber composition data were not provided ($n = 62$), (iii) pre- or post-intervention muscle fiber composition data were ambiguous ($n = 7$), (iv) muscle fiber composition data were provided for muscles other than the hind/lower limb ($n = 6$), (v) muscle fiber composition data provided focused on the effect

of aging ($n = 3$), (vi) the study provided a duplicated dataset ($n = 2$), and (vii) the source was secondary ($n = 23$). In addition, two studies quantified the change over time in fiber composition in persons with permanent loss of muscle innervation due to catastrophic injury^{257,258}. These studies were not included in the meta-analysis given their distinct difference from all other studies. The remaining 123 studies were published between 1973 and 2019. There were a total of 2,033 participants across these 123 studies, with a mean reported age and body mass of 32.9 ± 17.4 years and 72.8 ± 9.3 kg. The total number of participants in each intervention category were: bed rest ($n = 153$), immobilization ($n = 179$), environmental ($n = 30$), spaceflight ($n = 21$), endurance training ($n = 313$), strength training ($n = 1,018$), sprint training ($n = 92$), dynamic training ($n = 96$), and detraining ($n = 131$) (**Table 2.3**; see category definitions therein).

The effect of each intervention category on lower limb muscle slow fiber content ranged from $-9.3 \pm 6.9\%$ in spaceflight to $2.3 \pm 5.7\%$ in endurance training (**Table 2.3**; **Fig. 2.3a**). Comparisons based on the m. vastus lateralis alone were similar, albeit with a slightly smaller range ($-7.3 \pm 1.4\%$ to $2.4 \pm 6.4\%$; **Fig. 3B**). Overall, these pooled intervention effect sizes are substantially lower than the degree of difference observed between the hind/lower limb muscles of humans and chimpanzees ($31.1 \pm 9.6\%$) and humans and the gorilla ($31.3 \pm 9.6\%$). This is also the case when looking at the m. vastus lateralis alone (H v. C: $11.0 \pm 4.2\%$; H v. G: $17.1 \pm 4.2\%$).

Fig. 2.3 (next page). Average change in lower limb muscle slow fiber content across 9 human intervention categories compared to the human/African ape differential. (a) Analysis of studies of lower limb muscle slow fiber content before and after a range of bed rest, immobilization, spaceflight and exercise interventions. (b) Analysis of studies of *m. vastus lateralis* slow fiber content before and after a range of bed rest, immobilization, spaceflight and exercise interventions. Strength and endurance training increased slow fiber content, while most other activities decreased slow fiber content, on average.

A



B

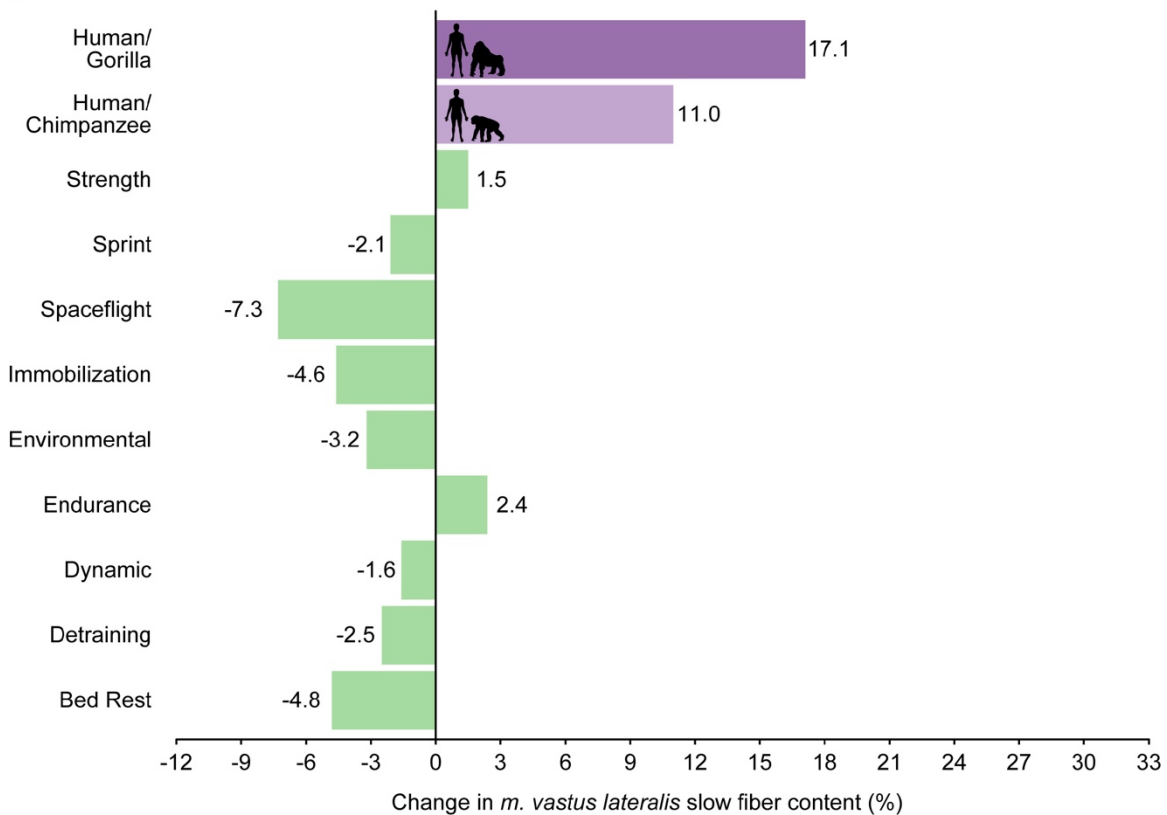


Table 2.3. Human intervention studies.

Intervention ^a	No. of studies	No. of subjects	Average Mass (kg)	Age (yrs)	Muscles ^b	Duration (days)	Weighted Delta (%) ^c	Standard Deviation	References
Bed rest	18	153	73.3	32.6	VL, SL	14-112	-5.6	9.4	234,259-275
Immobilization	11	179	74.6	30.2	VL, unknown leg m.	14-42	-3.9	3.5	276-286
Environmental	3	30	77.8	26.8	VL	21-40	-3.2	5.2	287-289
Spaceflight	3	21	76.1	42.0	VL, SL, GS	5-182	-9.3	6.9	290-292
Endurance training	25	313	71.1	29.4	VL, SL, GS, quadriceps, unknown leg m.	1-168	2.3	5.7	285,293-316
Strength training	58	1018	71.7	36.5	VL	14-365	1.5	4.4	281-283,309,311,314,315,317-367
Sprint training	10	92	74.8	30.3	VL	1-91	-2.1	6.8	161,300,308,368-374
Dynamic training	7	96	76.5	28.3	VL	70-1460	-1.6	4.5	309,311,314,375-378
Detraining	16	131	74.4	24.1	VL, GS	14-1460	-2.5	4.2	298,305,306,310,320,335,343,356,361,369,373,378-382

^a During bed rest the participants were confined to a bed at a hospital or research center and monitored continuously. During immobilization one of the participants' legs were prevented from weight-bearing to reduce muscle activity while participants continued their habitual daily activities. During environmental the participants were subjected to various living conditions (e.g., reduced barometric pressure or hypoxia) in a controlled research chamber for varying amounts of time with or without supine leg training. During spaceflight the participants (astronauts) flew aboard the International Space Station and followed an individually structured aerobic and resistance exercise countermeasure program. During endurance training the participants followed an aerobic training regimen, typically involving cycling or running exercises, aimed at increasing cardiovascular and/or muscular endurance. During strength training the participants followed various resistance training regimens, typically involving free weights and/or exercise machines, aimed at building muscle mass and improving muscle strength. During sprint training the participants followed a high intensity interval training regimen, typically involving a cycle ergometer, aimed at increasing cardiovascular health, muscle mass, and muscle power. During dynamic training the participants followed a combined endurance exercise and resistance training regimen. During detraining the participants ceased training/exercise after a period of vigorous training but otherwise continued their habitual daily activities.

^b Muscles measured before and after intervention. VL = vastus lateralis, SL = soleus, GS = gastrocnemius, quadriceps = undefined muscle of the quadriceps muscle group (v. lateralis, medialis, intermedius, r. femoris), unknown leg m. = undefined muscle of the leg.

^c Mean weighted delta = (control value – intervention value) · (no. of subjects in study / total no. of subjects per intervention)

2.4 Discussion

2.4.1 Humans MyHC I dominance is distinct from African apes and evolved within the hominin lineage

Humans are distinct from both gorillas and chimpanzees in the mean MyHC I isoform content of their pelvis and hind/lower limb skeletal muscles, while the two African apes are nearly identical across the same muscles. This reinforces the view that the predominance of MyHC I likely evolved within the hominin lineage¹⁰⁸, sometime since the *Pan-Homo* divergence 7 to 8 Ma¹⁰. This substantial human-African ape contrast appears robust whether young or old adult human samples are used, and is evident across pelvis, thigh, and leg muscles.

Across terrestrial mammals, skeletal muscle slow fiber content increases with body mass, albeit with substantial interspecific variation. This is consistent with an earlier assessment¹⁰⁸ and confirms that almost all terrestrial mammals studied to date are fast fiber dominant, on average. The chimpanzee and gorilla data indicate that African apes are most similar to most other terrestrial mammals in this regard, including other primates such as macaques, galagos and mouse lemurs. Human slow fiber dominance is therefore an exception to this pattern, which appears to be maintained in both young (22 yrs)¹⁶⁵ and old adults (83 yrs)¹⁶⁶. The absence of a significant age effect on slow fiber content is consistent with detailed cross-sectional studies of *m. vastus lateralis*, which previously reported a small, but nonsignificant increase from 10 to 85 yr olds^{383,384}. Still, there is marked variance in slow fiber content among individual muscles, with most of the muscles studied herein recruited during walking and running¹⁰⁸. Whether there are age-related shifts between fast (e.g., MyHC IIa and IIc) or between hybrid fibers (e.g., MyHC IIa/IIc) in humans requires further assessment.

The skeletal muscles of slow lorises (*Nycticebus coucang*) and sloths (*Bradypus variegatus*, *Choloepus hoffmanni*) are also slow fiber dominant on average, but this is almost certainly due to ecological factors that are distinct from those of humans. Whereas human walking and running capabilities likely benefit from an enhanced capacity for repetitive, low-cost contractile behavior, neither the slow loris nor the sloth have much need for frequent, overground travel. Instead, their commonalities may reflect a shared reliance on fatigue-resistant, low-force contractile behavior, used in the context of cryptic and 'slow climbing' (slow loris)³⁸⁵⁻³⁸⁷ or extended tree hanging (sloth)^{388,389}.

2.4.2 Human MyHC I content is an evolved trait with modest responsiveness to behavioral interventions

A -9 to 2% shift in muscle slow fiber content in response to a wide range of behavioral interventions is quite modest when compared to the average 31% interspecific differences between humans and African

apes (**Fig. 2.3A, Table 2.2**)¹⁰⁸. There is of course variance among individuals in average lower limb slow fiber content, with Johnson et al.¹⁶⁵ exhibiting less between-subjects variance than Tirrell et al.¹⁶⁶ for the same number of individuals (i.e., J: s.d. = 5.7%; T: s.d. = 13.7%; **Fig 2.1B**). Yet, neither of these human datasets overlap the chimpanzee or gorillas. This is important since the average MyHC I shifts reported here control for between-subject or between-muscle variance by sampling longitudinal studies alone, in contrast to cross-sectional comparisons (e.g., sprinters v. marathoners) that have the potential to be misleading in regard to responsiveness. Indeed, these results imply that the human-African ape contrast in MyHC I content is an evolved trait, not simply a reflection of increased or decreased activity level during a lifetime – distinct from the level of plasticity that influences muscle size^{220,221} or even some skeletal parameters (e.g., cortical bone distribution)³⁹⁰.

Among the 2,033 subjects and 9 behavioral groups studied to date, only endurance training (e.g., running or cycling) and strength training (e.g., weightlifting) are associated with an increase in skeletal muscle slow fiber content, although the magnitude of this effect is quite small (i.e., 1.5-2.3%, **Table 2.3**). Longitudinal comparisons of different behavioral interventions in other taxa (e.g., mouse models) may provide additional insights into this pattern; however, most model investigations are cross sectional. All other behavioral interventions decrease skeletal muscle slow fiber content, on average. This is important since the African ape, macaque and human data indicate that the shift within the hominin lineage was an increase in MyHC I isoform content, regardless of whether the last common ancestor of the *Pan* and *Homo* lineages was more similar to African apes or a generalized arboreal ape in its locomotor behavior^{391,392}.

The timing of the shift toward MyHC I dominant skeletal muscle within the hominin lineage is difficult to establish. However, we anticipate this shift to have been concurrent with major transitions in locomotor behavior, daily travel distance, home range size or some combination thereof. The earliest australopithecines (e.g., *Australopithecus anamensis*, *Australopithecus afarensis*) exhibit changes to the pelvis and lower limb skeleton as compared to *Ardipithecus ramidus*^{18,30,393}, suggesting an adaptive shift towards greater overground locomotion as early as 4 Ma, at least in east Africa. Between 2.1 and 1.8 Ma, *Homo erectus* appears in east and south Africa^{394,395}, east Europe³⁹⁶ and east Asia^{397,398}, indicating a global dispersal that must have involved substantial overground walking and running. This species would seem to benefit from a lower-cost, fatigue-resistant skeletal muscle design, although an earlier evolutionary shift or multiple, incremental shifts as part of ongoing selection for overground locomotion in the hominin lineage is also plausible. A better understanding of the molecular mechanisms that determine adult muscle MyHC content in primates and other mammals may provide additional information on the initial appearance, pleiotropic effects, or selective pressures acting on this trait within the hominin lineage.

2.4.3 *Enhancers may play an important role in hominin skeletal muscle evolution*

Our findings indicate that muscle fiber content is likely an evolvable trait that was under selection in the hominin lineage. MyHC isoforms expressed in mammalian skeletal muscle are encoded by specific MyHC genes¹³³. While these genes, by nature of the proteins they encode, represent obvious candidates for selection in the hominin lineage, they are highly conserved across mammals³⁹⁹ and likely under heavy evolutionary constraint across species. Indeed, mutations in MyHC genes can have multiple effects (i.e., be pleiotropic) and are associated with human disease^{400–402}. Despite this, MyHC gene expression varies considerably across tissues (e.g., skeletal, cardiac, extraocular, and masticatory muscle), time points (e.g., embryonic, fetal, adult), and taxonomic lineages (e.g., small and large mammals)^{159,160,399}. As such, regions of the genome that regulate the expression of these genes may better explain observed variation in slow fiber content across mammals (**Fig. 2.2; Appendix B; Appendix C**) and make compelling candidates for understanding the shift in MyHC content in hominin skeletal muscle.

Indeed, phenotypic differences between humans and chimpanzees have long been attributed to species-specific mutations in non-coding regulatory regions of the genome¹⁷², which influence both where and when genes are expressed. Mutations in regulatory elements can change the spatiotemporal expression of genes during growth and development and lead to phenotypic variation and evolutionary divergence between closely related species^{193,403–414}. For example, the shapes of the human knee and pelvis, compared to those of African apes, have resulted from evolutionary selection on regulatory sequences unique to the individual skeletal elements of the knee (i.e., distal femur)⁴¹³ and pelvis (i.e., ilium)⁴¹³. In line with these studies, here we hypothesize that natural selection has selected for human-specific variation in the non-coding regions of the genome that regulate the expression of fast MyHC II and slow MyHC I genes, rather than the MyHC genes themselves. The result of this selection would be increases in the amount of fatigue-resistant slow MyHC I muscle fibers in human lower limb muscles to facilitate repetitive, low-cost contractile behavior during walking and running.

Of particular interest are enhancers, a type of non-coding regulatory element that possess remarkable specificity in their control of gene expression. Enhancers can regulate gene expression in a tissue-specific or developmental stage-specific manner, leading to modularized effects on phenotype¹⁷³. Previous research has also demonstrated that species-specific mutations within conserved enhancer regions can lead to evolutionary novelty^{176,405,410,415,416} or convergence^{174,417–419}. MyHC “super enhancers” have recently been identified in mouse skeletal muscle that regulate the expression of genes belonging to the fast and slow MyHC gene clusters^{195,196}. Given that the temporal regulation of MyHC genes during development is critical for the stage-specific progression of primary myotubes to developmental (i.e., embryonic and fetal) and adult muscle fibers¹³³, and the replacement of developmental isoforms (i.e., MyHC-emb and MyHC-neo) with adult slow (MyHC I) and fast (MyHC II) isoforms^{223,224}, it is likely that

species-specific differences in adult MyHC content, such as those observed between humans and African apes, are due to species-specific changes in MyHC enhancers (**Fig. 2.4**). Similarities in adult MyHC content, as seen in humans, sloths and slow lorises, may be due to functionally equivalent mutations in these evolutionarily conserved MyHC enhancers, although trait convergence may also have arisen via the evolution of novel, species-specific enhancers^{181,408,420–422}. A more comprehensive consideration of the molecular mechanisms involved in regulating muscle MyHC isoform content in humans and other mammals is needed to refine this understanding of skeletal muscle evolution and to reconstruct how and when these changes occurred.

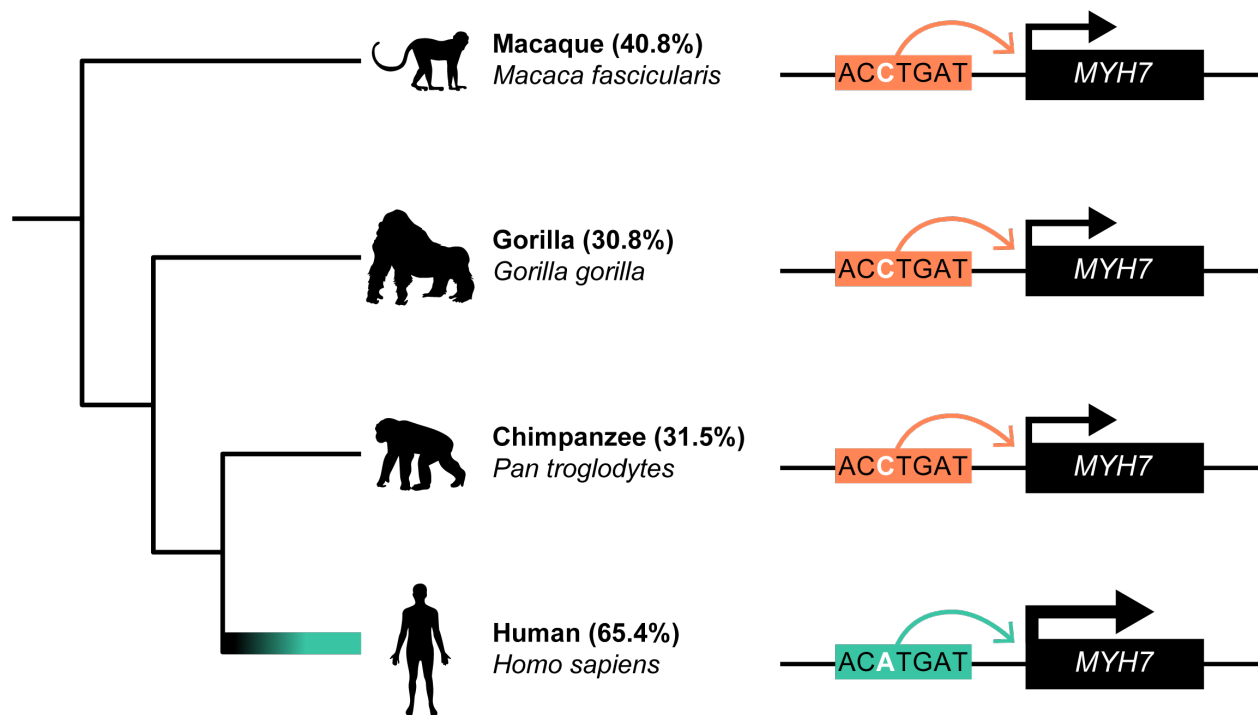


Fig. 2.4. One hypothesized regulatory mechanism underlying the observed variation in pelvis and hind/lower limb MyHC I content across species and human slow fiber dominance. The average, unmatched pelvis and hind/lower limb muscle slow fiber content for each species is given in parentheses. Here, a conserved enhancer (highlighted in orange or green) is shown to regulate the expression of *MYH7*, the gene encoding slow MyHC I. The non-human primates (orange enhancer) have identical DNA sequences within this conserved enhancer, reflecting similarities in muscle MyHC I content. Under this scenario, the hominin lineage acquired a mutation (or mutations) within a conserved enhancer (here represented in the green enhancer by a single C to A base pair change) after the *Pan-Homo* divergence, increasing the expression of *MYH7* and the proportion of slow MyHC I fibers within hominin pelvis and lower limb muscles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5 Conclusions

Humans are distinct from African apes (chimpanzees and gorillas) and among a small group of terrestrial mammals whose pelvis and hind/lower limb skeletal muscle is slow fiber dominant, on average.

Behavioral interventions, including immobilization, bed rest, spaceflight and exercise, can induce modest decreases and increases in human slow fiber content (i.e., -9.3% to 2.3%, $n = 2,033$ subjects), but these shifts are much smaller than the mean human-African ape differential. The relative magnitude of the human-African ape differential in MyHC I content suggests that selection in the hominin lineage for low-cost, repetitive contractile behavior may underlie this difference. As a result, greater characterization of the regulatory landscape of muscles during muscle fiber development between species with divergent MyHC isoform composition is needed to reconstruct the evolutionary history of observed shifts in human skeletal muscle MyHC content.

2.6 Bridge

Chapter 2 situated humans within a broader mammalian context, asking how “unique” human muscles truly are and whether human behavior (e.g., exercise) could make them appear more “chimpanzee-like” (i.e., fast myofiber-biased). We show that larger-bodied species generally exhibit higher proportions of slow myofibers, yet humans fall within a small subset of mammals with far more slow myofibers than predicted by size. In addition, our data indicate that muscle myofiber content is a fixed, genetically determined, species-level trait established during development and maintained throughout life, as normal human activity (e.g., running, weightlifting) does not induce shifts large enough to overcome the difference in slow myofiber content between humans and chimpanzees. Chapter 3 builds upon fixed species differences in myofiber composition, providing broader context for the mammalian myofiber dataset, identifying avenues for further interspecific comparisons, and highlighting the need for histochemical data from underrepresented species and muscles. Given the genetic basis of muscle myofiber composition, Chapter 4 then turns to identifying the genetic mechanisms that regulate myofiber type as a first step toward understanding human muscle evolution.

3 META-ANALYSIS OF SKELETAL MUSCLE SLOW FIBER CONTENT ACROSS MAMMALIAN SPECIES

From Queeno, S. R., Sterner, K. N., & O'Neill, M. C. (2023). Meta-analysis data of skeletal muscle slow fiber content across mammalian species. *Data in Brief*, 50, 109520.

3.1 Introduction

Herein, the dataset generated for Chapter 2 is presented and described. Mammalian skeletal muscle slow (MyHC-I) fiber composition data was collated from 268 eligible studies identified via a systematic literature search and meta-analysis, following a structure similar to PRISMA²²⁶. Academic search systems were queried with terms relating to mammalian skeletal muscle fiber content and reference lists of selected articles were thoroughly investigated for additional studies. Eligible studies were those that provided skeletal muscle fiber composition data from mammalian species that were not subjected to experimental manipulations. Taxonomic information, sex, age, number of individuals sampled, average body mass (kg), average slow fiber content (%) of each skeletal muscle under investigation and fiber-typing methodology were collated from eligible studies when available. Muscle fiber composition data was collected from more than 200 skeletal muscles across 174 mammalian species, which will be of value to those interested in muscle physiology, interspecific muscle comparisons, and connections between muscle physiology, taxonomy, body mass, ecomorphology and locomotor strategy (among others).

3.2 Value of the data

- This is the first meta-analysis of its kind, which compiles skeletal muscle fiber composition data across 174 mammalian species into a single, usable file.
- These data will be of value to scientists interested in muscle physiology, interspecific muscle comparisons, and connections between muscle physiology, taxonomy, body mass, ecomorphology and locomotor strategy (among others).
- These data highlight certain species, taxonomic orders, and muscles for which fiber composition data is lacking and needs investigation.
- These data will spark interest in gathering muscle fiber composition data from currently unsampled (or underrepresented) species and muscles, generate interest in pursuing questions relating to muscle physiology and evolution, as well as analyses based on interspecific datasets.

3.3 Objective

Skeletal muscle slow fiber content varies across muscles and taxa and is one of the traits that distinguishes humans from other apes^{423,424}, yet no study to date has compiled these data into a single, usable format. The goal of this study was to compile mammalian skeletal muscle slow (MyHC-I) fiber

composition data from published, peer-reviewed articles for interspecific comparison and analysis.

3.4 Data description

3.4.1 Figure 3.1

A total of 2,610 studies were found from academic search systems (Google Scholar, PubMed, and JSTOR) and library databases according to the selection criteria (**Fig. 3.1**). Of these, 2,459 studies were selected for screening. After reading the abstract, 388 articles were selected for full-text eligibility assessment. In total, 268 studies fully met the selection criteria and were selected for inclusion in the meta-analysis.

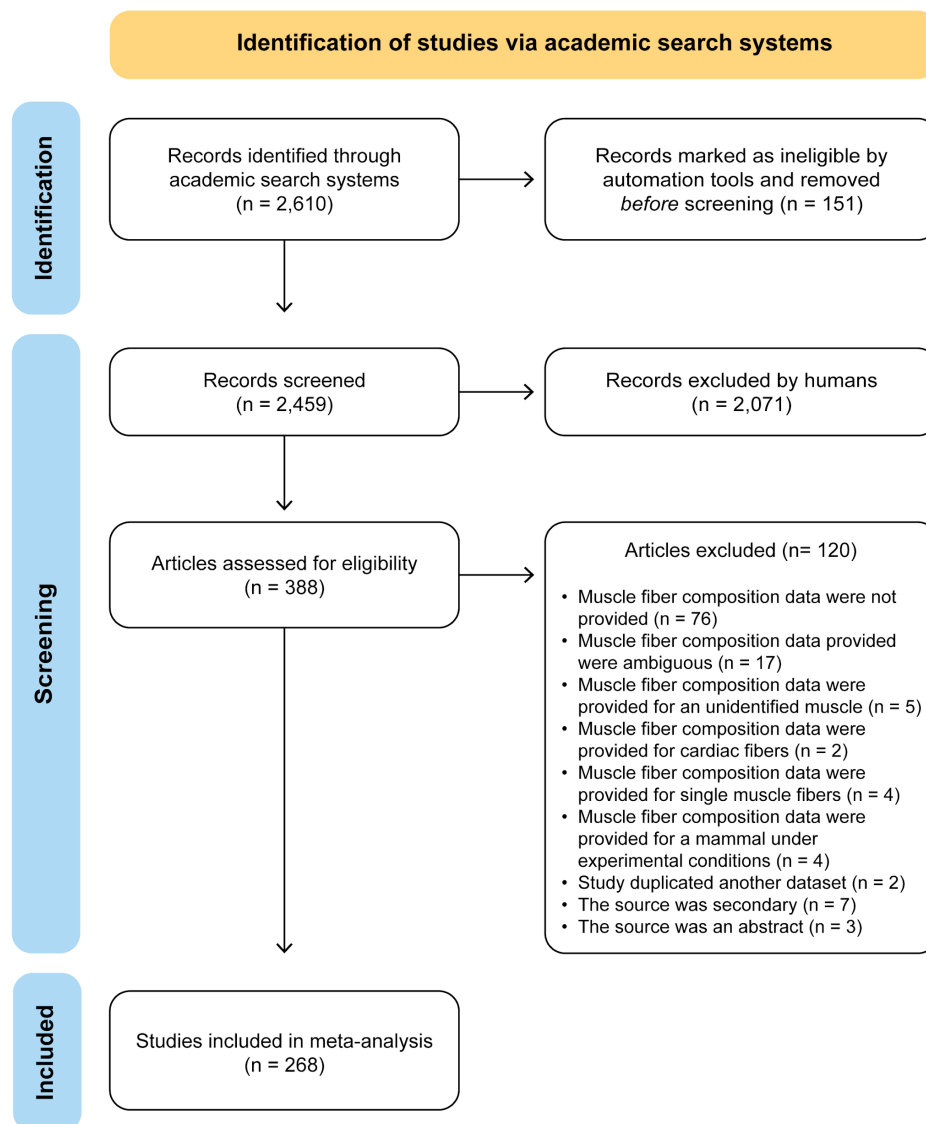


Fig. 3.1. PRISMA flow diagram describing the systematic review and meta-analysis workflow.

3.4.2 Figure 3.2

The 268 eligible studies included in the meta-analysis were published between 1965 and 2023 (Fig. 3.2). Interest in, and ability to, determine the fiber composition of different skeletal muscles from various mammalian species increased in the 1970s. The earliest included study to use the histochemical assay for myofibrillar ATPase activity (mATPase) to determine fiber type across multiple skeletal muscles was published in 1969¹⁶⁸. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was first used in 1981^{217,425}, and immunohistochemistry with myosin antibodies (mABs) was first used in 1990⁴²⁶. The first RNA-based method to determine skeletal muscle fiber composition, reverse transcription-polymerase chain reaction (RT-PCR), was first used in 2003⁴²⁷.

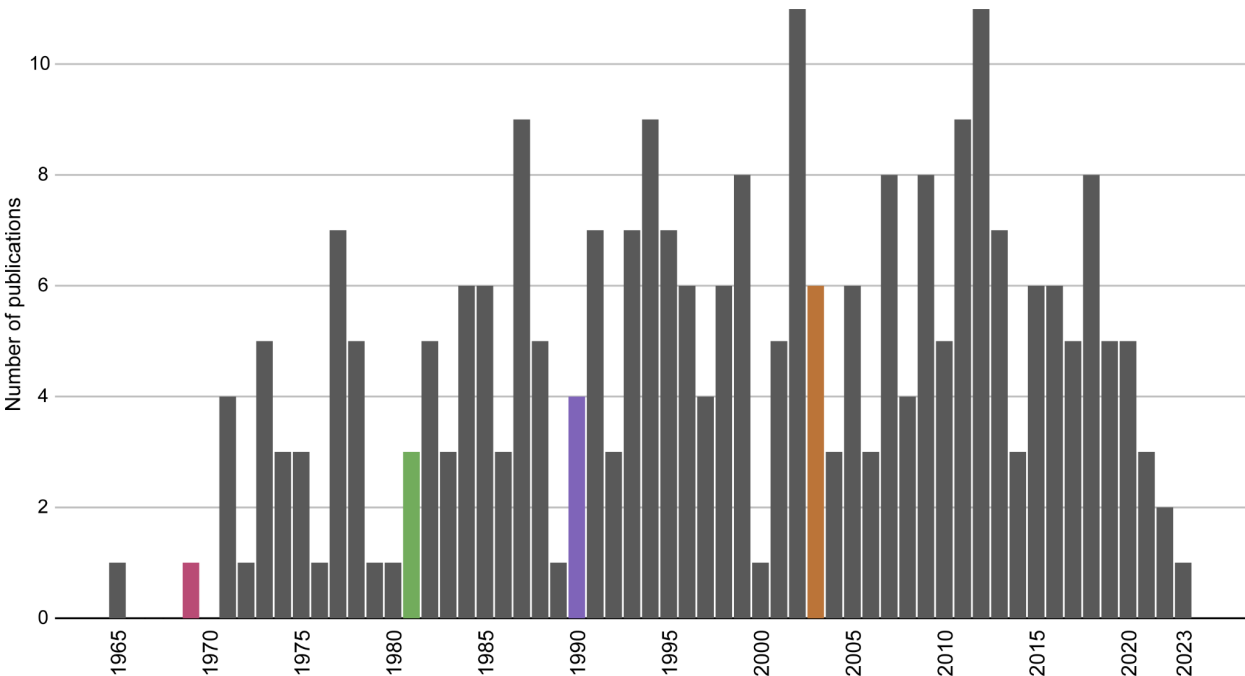


Fig. 3.2. Number of mammalian skeletal fiber composition publications included in the meta-analysis per year. Colored bars denote the first appearance of each fiber-typing method: mATPase (raspberry), SDS-PAGE (green), mABs (purple), and RT-PCR (orange).

3.4.3 Table 3.1

Table 3.1 is the dataset behind Fig. 3.3 and Fig. 3.4.

Table 3.1 (next page). Taxonomic composition, diversity, and representation of each of the 15 mammalian orders present in the 268 eligible studies.

Order	No. of families	No. of genera	No. of species	No. of publications
Artiodactyla	9	21	26	53
Carnivora	8	17	22	36
Chiroptera	6	12	16	8
Cingulata	1	1	1	1
Dasyuromorphia	1	1	1	1
Didelphimorphia	1	3	3	4
Diprotodontia	2	5	8	4
Eulipotyphla	2	7	10	6
Lagomorpha	2	2	2	7
Perissodactyla	1	1	3	13
Pilosa	2	2	2	3
Primates	11	35	50	64
Proboscidea	1	1	1	1
Rodentia	10	23	26	66
Scandentia	1	1	3	1

3.4.4 Figure 3.3

From these 268 eligible studies, skeletal muscle fiber composition data was collated from 174 species belonging to class Mammalia (**Table 3.1**). These species represent 15 unique taxonomic orders, 58 families, and 132 genera.

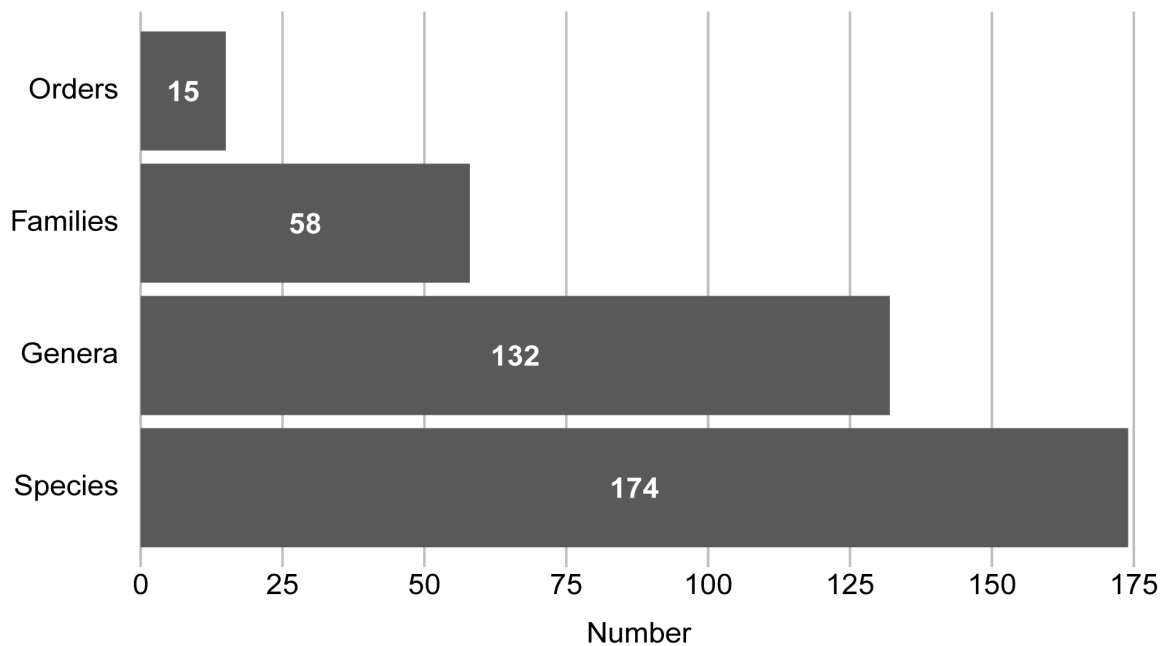


Fig. 3.3. Taxonomic representation across the 268 eligible mammalian fiber composition studies included in the meta-analysis.

3.4.5 Figure 3.4

Within class Mammalia, orders Rodentia, Primates, and Artiodactyla are the most highly represented in the literature, whereas orders Cingulata, Dasyuromorphia, Proboscidea, and Scandentia are the least represented (**Fig. 3.4A**). Similarly, orders Rodentia, Primates, and Artiodactyla have the most diverse representation in the literature in terms of number of unique families (**Fig. 3.4B**), genera (**Fig. 3.4C**), and species (**Fig. 3.4D**).

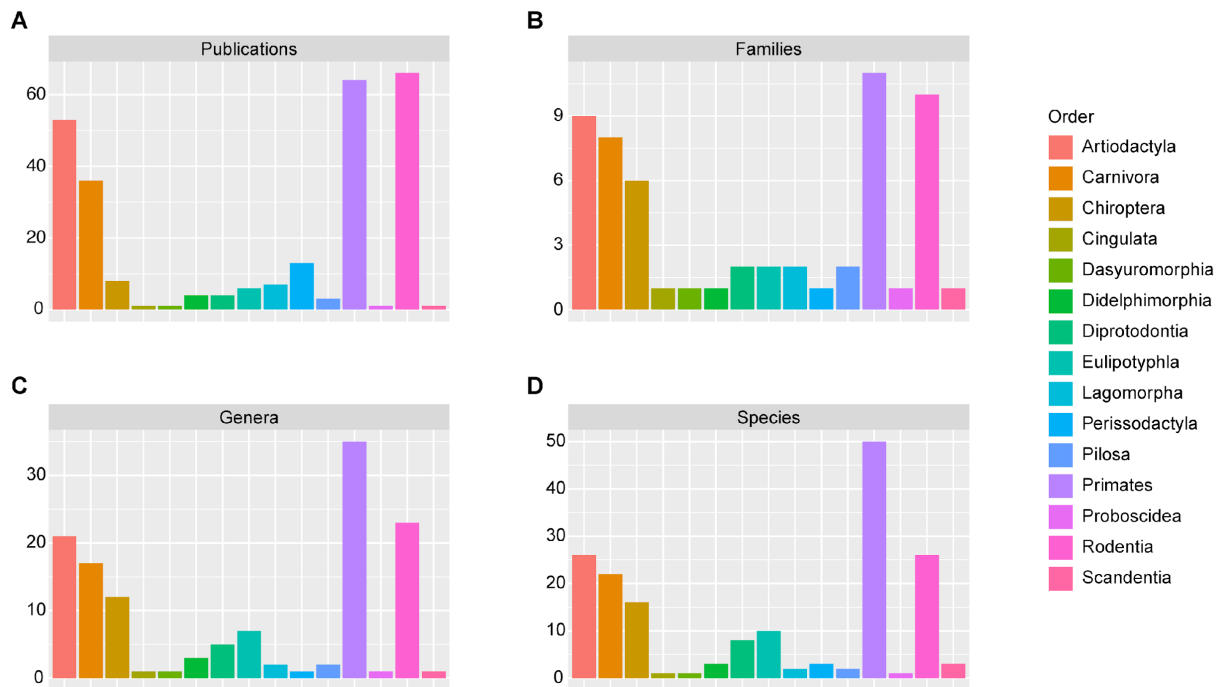


Fig. 3.4. Study representation and taxonomic diversity across the 15 mammalian orders. (a) Number of eligible studies/publications per taxonomic order. (b) Number of taxonomic families per taxonomic order. (c) Number of genera per taxonomic order. (d) Number of species per taxonomic order.

3.4.6 Table 3.2

Table 3.2 quantifies the number of studies used in the meta-analysis that did not report the number of individuals from which skeletal muscle fiber composition data was recorded ($n = 22$) or the body mass of the individuals from which skeletal muscle fiber composition data was recorded ($n = 136$). Sixteen of the 268 eligible studies reported neither the number of individuals nor the body mass of individuals from which skeletal muscle fiber composition data was recorded.

Table 3.2 (next page). Number of eligible studies missing relevant data for meta-analysis.

Variable	No. of publications
Did report number of individuals sampled	246
Did not report number of individuals sampled	22
Did report body mass of sampled individuals	132
Did not report body mass of sampled individuals	136
Did not report either number of individuals sampled or body mass of sampled individuals	16

3.4.7 Figure 3.5

Fourteen species across 6 taxonomic orders had skeletal muscle fiber composition data from 50 or more individuals (**Fig. 3.5**). Five species had skeletal muscle fiber composition data from 100 or more individuals: pigs ($n = 825$), humans ($n = 338$), horses ($n = 193$), rats ($n = 168$), and cats ($n = 108$).

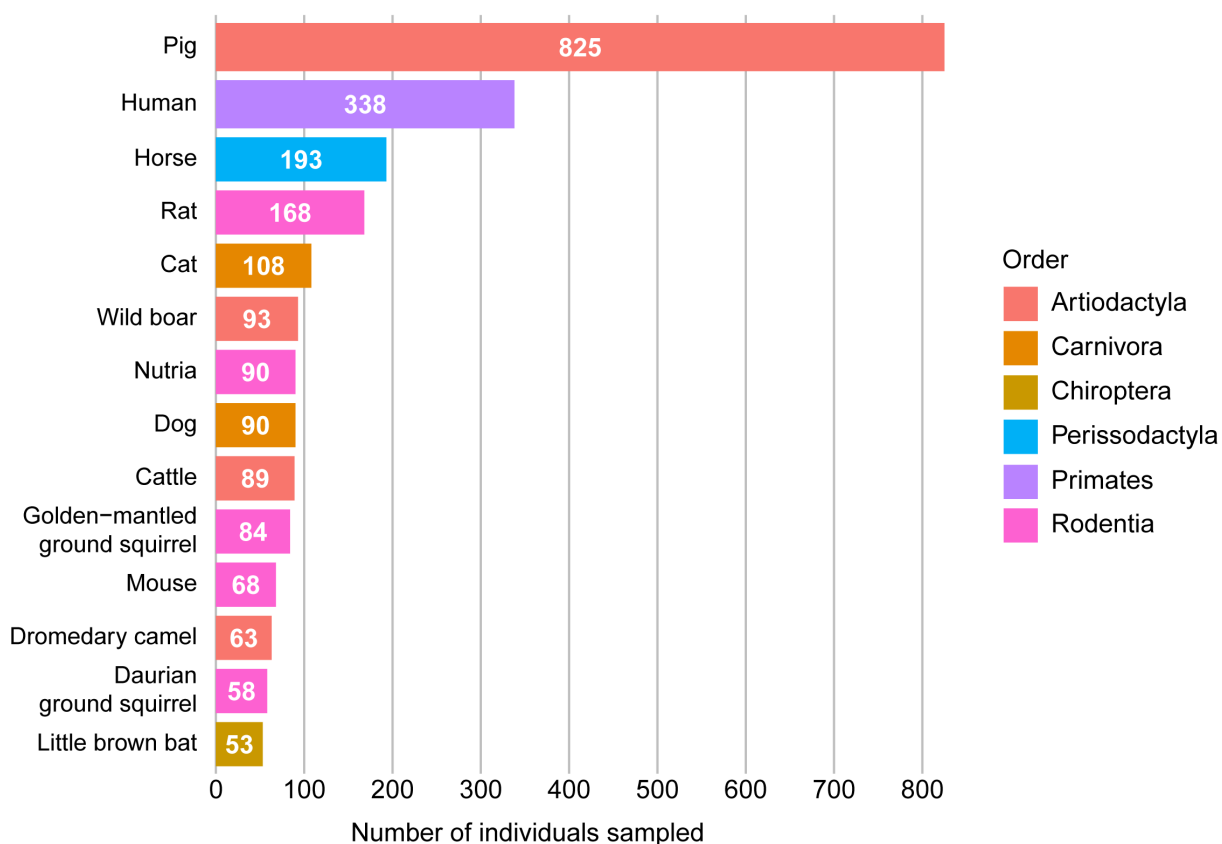
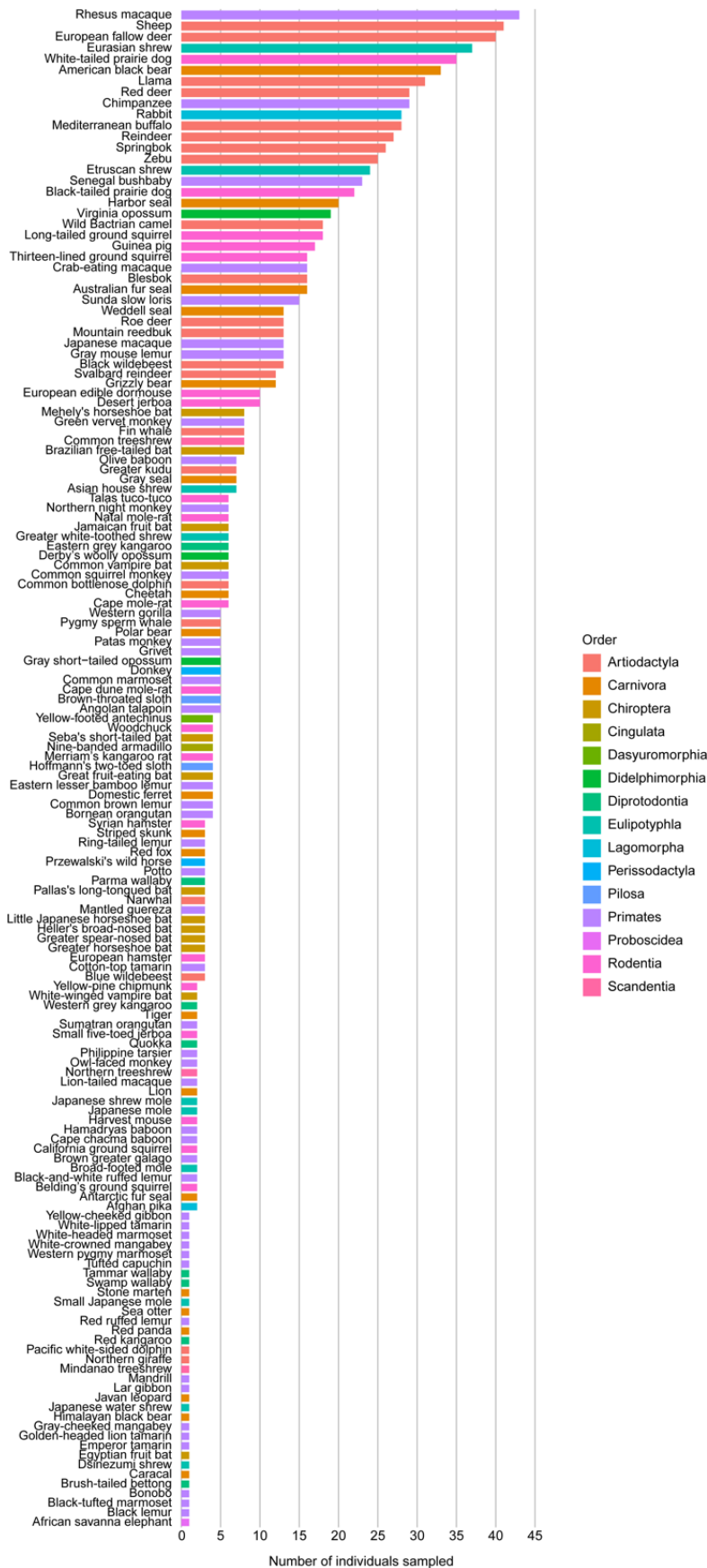


Fig. 3.5. Species with greater than 50 sampled individuals.

3.4.8 *Figure 3.6*

154 species had skeletal muscle fiber composition data from 50 or fewer individuals (**Fig. 3.6**). Of those, 33 species had skeletal muscle fiber composition data from only one individual: African savanna elephant, black lemur, black-tufted marmoset, bonobo, brush-tailed bettong, caracal, Dsinezumi shrew, Egyptian fruit bat, emperor tamarin, golden-headed lion tamarin, gray-cheeked mangabey, Himalayan black bear, Japanese water shrew, Lar gibbon, mandrill, Mindanao treeshrew, northern giraffe, Pacific white-sided dolphin, red kangaroo, red panda, red ruffed lemur, sea otter, small Japanese mole, stone marten, swamp wallaby, Tammar wallaby, tufted capuchin, western pygmy marmoset, white-crowned mangabey, white-headed marmoset, white-lipped tamarin, and yellow-cheeked gibbon.

Fig. 3.6 (next page). Species with fewer than 50 sampled individuals.



3.4.9 Figure 3.7

The 174 species included in the meta-analysis ranged in average body mass from 0.0019 kg (bumblebee bat, *Craseonycteris thonglongyai*) to 70,000 kg (fin whale, *Balaenoptera physalus*) (**Fig. 3.7**).

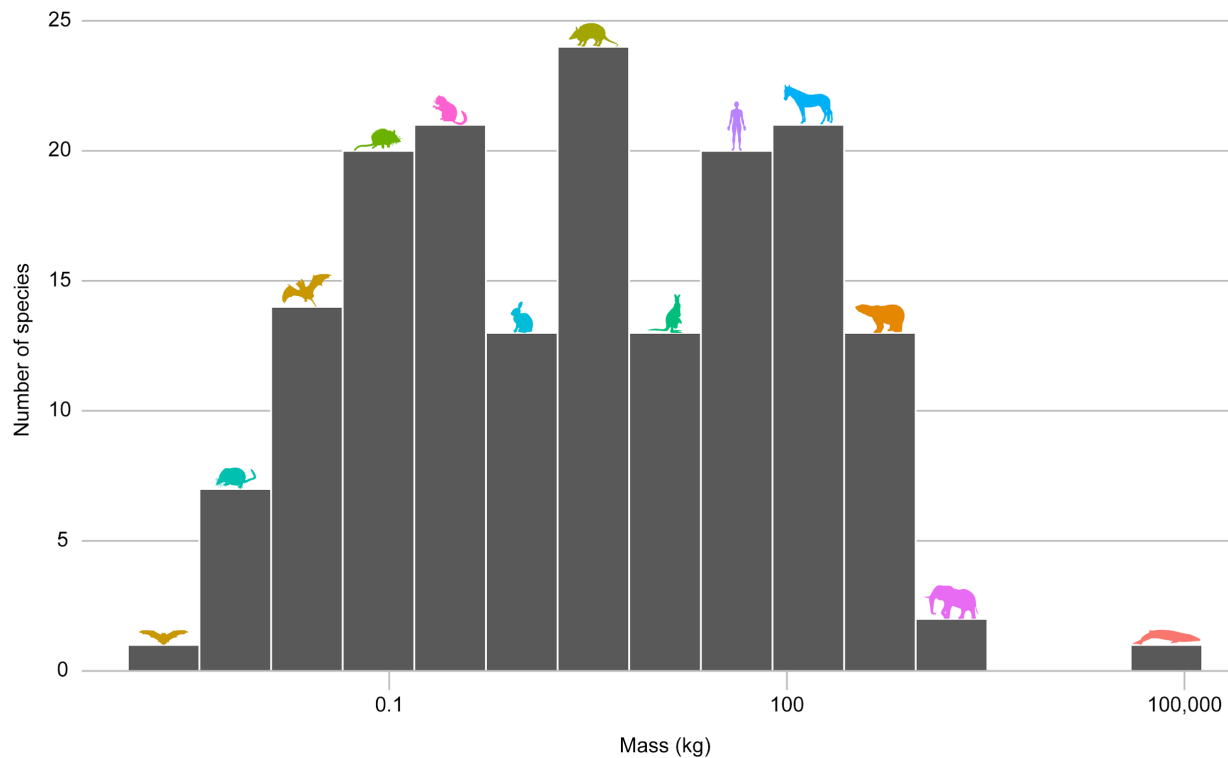


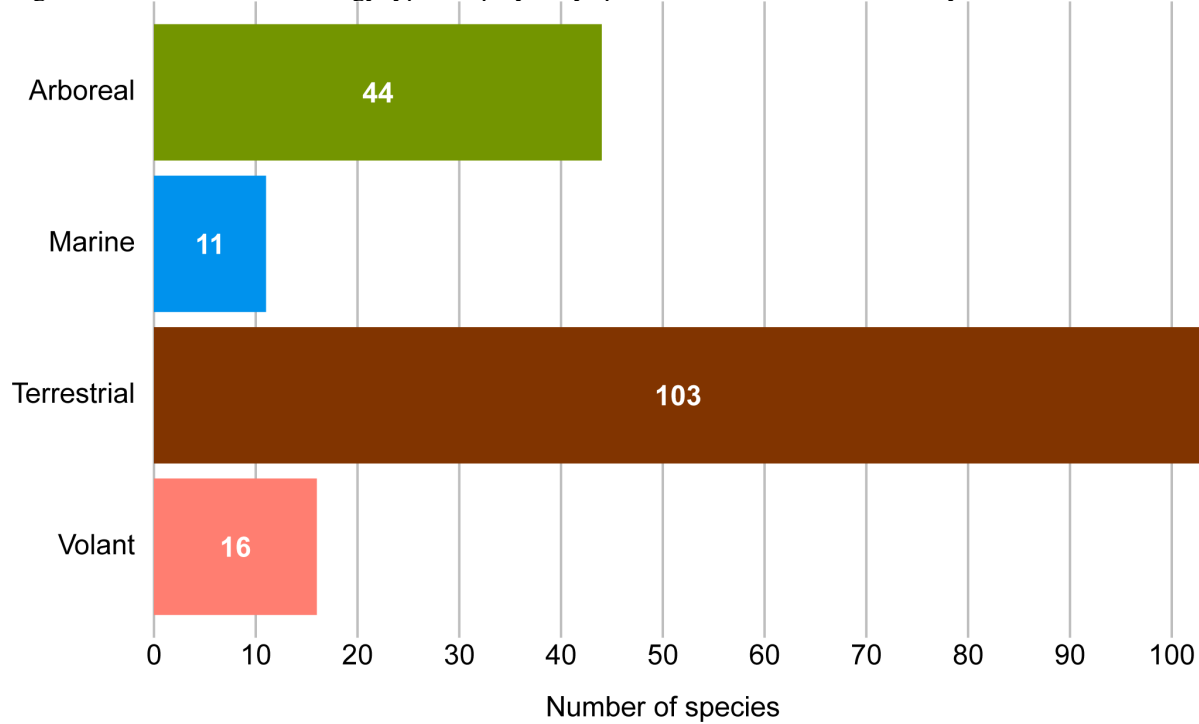
Fig. 3.7. Body mass range of mammalian species included in the meta-analysis. Icons represent some of the species included within each mass bin and are colored by taxonomic order.

3.4.10 Figure 3.8

Broad locomotor strategies employed by species included in the meta-analysis (**Fig. 3.8**). Most species providing skeletal muscle fiber composition data are terrestrial ($n = 103$), whereas marine ($n = 11$) species are the least represented. Arboreal species are those that spend most of the time in the trees and locomote via arboreal quadrupedalism, vertical clinging and leaping, brachiation, suspension, and other scansorial activities. Marine species are those that spend most of the time in the ocean and locomote via swimming, diving, and other natatorial activities. Terrestrial species are those that spend most of the time on the ground and locomote via terrestrial quadrupedalism, bipedalism, or saltation, and may be fossorial, semi-fossorial, cursory, ambulatory, graviportal, generalized, amphibious, or scansorial. Volant

species are those that primarily locomote via powered flight.

Fig. 3.8. Broad locomotor strategy types employed by species included in the meta-analysis.



3.4.11 Table 3.3

Skeletal muscle fiber composition data was provided for 238 muscles across 11 broad anatomical compartments: head, neck, shoulder, brachium (i.e., upper arm), antebrachium (i.e., lower arm), hand, trunk (i.e., back and abdominal musculature), pelvic/gluteal, thigh, leg and tail. **Table 3.3** quantifies the number of unique skeletal muscle sampling terms (excluding differences in anatomical sampling location from the same muscle such as “superficial,” “deep,” “proximal,” “distal,” etc.) per anatomical compartment, as well as the number of data points per anatomical compartment. The anatomical compartment with the greatest number of unique skeletal muscle sampling terms is the trunk ($n = 41$). The most heavily sampled anatomical compartments are the leg ($n = 684$) and thigh ($n = 661$), followed by the shoulder ($n = 340$) and trunk ($n = 331$).

Table 3.3. Number of unique skeletal muscle sampling terms and data points per anatomical compartment.

Anatomical compartment	No. of unique skeletal muscle sampling terms	No. of data points
Head	21	115
Neck	20	46
Shoulder	17	340

Anatomical compartment	No. of unique skeletal muscle sampling terms	No. of data points
Brachium	12	238
Antebrachium	21	105
Hand	16	21
Trunk	41	331
Pelvic/gluteal	27	210
Thigh	27	661
Leg	23	684
Tail	13	27
Total	238	2,778

3.4.12 Table 3.4

Sampling richness and taxonomic diversity across skeletal muscles from which fiber composition data was recorded (**Table 3.4**). Table 3.4 quantifies the number of data points, sampled individuals, sampled species, and studies per skeletal muscle, as well as the average (unweighted) slow fiber composition of each sampled skeletal muscle across species. Data from Table 3.4 is used for **Figure 3.9** and **Figure 3.10**.

Table 3.4. Sampling characteristics and average (unweighted) slow fiber composition of each sampled skeletal muscle included in the meta-analysis. If the number of individuals sampled was not reported in the study, a value of “NA” is given in the “No. of individuals” column. Broad muscle terms marked as “undefined” are those from the literature that did not identify the specific muscle bellies included in the sample.

Muscle	Anatomical compartment	No. of data pts	No. of individuals	No. of species	No. of studies	Avg. (%) slow
Buccinator	Head	1	6	1	1	13.4
Cheek pouch (undefined)	Head	1	3	1	1	0.0
Cheek pouch retractor	Head	1	3	1	1	15.0
Depressor conchae	Head	1	10	1	1	30.0
Digastricus (anterior)	Head	10	18	10	1	9.0
Digastricus (posterior)	Head	1	7	1	1	2.3
Extraocular	Head	1	NA	1	1	0.0
Frontalis	Head	1	6	1	1	64.1
Masseter	Head	26	94	21	13	19.9
Medial pterygoid	Head	10	17	10	1	22.2
Mylohyoid	Head	12	21	12	3	20.5
Nasorostralis superficialis	Head	1	4	1	1	52.9
Orbicularis oculi	Head	3	21	2	3	12.5
Orbicularis oris	Head	1	10	1	1	15.0
Orbicularis oris (marginal)	Head	1	5	1	1	34.0
Orbicularis oris (peripheral)	Head	1	5	1	1	27.0
Styloglossus	Head	2	11	2	1	33.8
Temporalis	Head	18	50	14	7	16.9

Muscle	Anatomical compartment	No. of data pts	No. of individuals	No. of species	No. of studies	Avg. (%) slow
Tensor tympani	Head	10	17	10	1	21.5
Tensor veli palatini	Head	10	17	10	1	15.0
Tongue (undefined)	Head	3	NA	2	2	0.0
Biventer cervicis	Neck	2	9	2	2	52.2
Brachiocephalicus	Neck	2	3	2	2	37.7
Cleidocephalicus	Neck	2	15	2	2	17.9
Cleidocervicalis	Neck	1	3	1	1	24.0
Cleidomastoid	Neck	1	3	1	1	23.0
Cleidooccipital	Neck	1	6	1	1	28.0
Cricopharyngeus	Neck	2	16	2	2	40.8
Obliques capitis	Neck	1	6	1	1	39.0
Omotransversarius	Neck	4	34	4	4	32.7
Rectus capitis posterior major	Neck	2	12	2	2	39.2
Rectus capitis posterior minor	Neck	1	6	1	1	23.0
Rhomboideus capitis	Neck	1	6	1	1	56.0
Scalenus	Neck	3	12	3	3	23.0
Semispinalis capitis	Neck	5	21	5	5	45.2
Semispinalis cervicis	Neck	1	6	1	1	38.0
Splenius	Neck	7	29	7	7	33.8
Sternocephalicus	Neck	3	12	3	3	29.2
Sternohyoid	Neck	3	13	3	3	21.9
Sternomastoid	Neck	3	19	3	3	28.0
Thyroarytenoideus	Neck	1	8	1	1	2.5
Atlantoscapularis	Shoulder	2	10	2	2	32.4
Cleidobrachialis	Shoulder	2	12	2	2	20.2
Deltoid	Shoulder	57	244	42	23	30.1
Dorsoepitrochlearis	Shoulder	1	3	1	1	28.0
Infraspinatus	Shoulder	31	152	25	19	27.7
Latissimus dorsi	Shoulder	29	102	24	22	30.7
Pectoralis	Shoulder	57	243	51	33	21.4
Pectoralis abdominis lateralis	Shoulder	1	4	1	1	38.3
Rhomboid	Shoulder	16	63	14	14	39.1
Spinodeltoidius	Shoulder	1	3	1	1	2.0
Spinotrapezius	Shoulder	2	7	2	2	42.3
Subclavius	Shoulder	1	6	1	1	23.3
Subscapularis	Shoulder	23	65	22	13	21.4
Supraspinatus	Shoulder	51	137	23	20	33.2
Teres major	Shoulder	30	87	29	16	27.3
Teres minor	Shoulder	7	26	6	6	44.3
Trapezius	Shoulder	29	151	25	19	30.1
Biceps brachii	Brachium	42	208	30	21	24.6
Biceps brachii (long)	Brachium	9	43	7	7	41.8
Biceps brachii (short)	Brachium	7	34	5	6	26.7
Brachialis	Brachium	22	80	21	13	30.4
Coracobrachialis	Brachium	5	17	4	5	41.3

Muscle	Anatomical compartment	No. of data pts	No. of individuals	No. of species	No. of studies	Avg. (%) slow
Tensor fascia antebrachii	Brachium	2	10	2	2	32.1
Triceps brachii	Brachium	42	250	20	17	31.7
Triceps brachii accessorius	Brachium	1	3	1	1	90.0
Triceps brachii (angular)	Brachium	1	4	1	1	24.5
Triceps brachii lateralis	Brachium	32	104	29	21	19.7
Triceps brachii longus	Brachium	44	187	33	24	25.7
Triceps brachii medialis	Brachium	31	113	25	20	42.5
Abductor pollicis longus	Antebrachium	2	9	2	2	43.2
Anconeus	Antebrachium	3	18	3	3	87.6
Brachioradialis	Antebrachium	9	43	6	7	40.4
Extensor carpi radialis	Antebrachium	7	34	6	7	26.0
Extensor carpi ulnaris	Antebrachium	5	24	5	5	38.8
Extensor digiti minimi (quinti)	Antebrachium	2	7	2	2	59.6
Extensor digitorum communis	Antebrachium	18	84	16	12	24.0
Extensor digitorum lateralis	Antebrachium	2	5	2	2	16.7
Extensor indicis proprius	Antebrachium	1	6	1	1	57.7
Extensor pollicis brevis	Antebrachium	1	6	1	1	57.8
Extensor pollicis longus	Antebrachium	1	6	1	1	50.4
Flexor carpi radialis	Antebrachium	5	31	5	5	29.2
Flexor carpi ulnaris	Antebrachium	11	59	10	10	38.6
Flexor digitorum communis	Antebrachium	3	11	3	3	27.8
Flexor digitorum profundus	Antebrachium	15	73	13	9	21.5
Flexor digitorum superficialis	Antebrachium	8	55	7	8	40.2
Flexor pollicis longus	Antebrachium	1	6	1	1	44.1
Palmaris longus	Antebrachium	4	21	4	3	43.9
Pronator quadratus	Antebrachium	2	9	2	2	56.8
Pronator teres	Antebrachium	3	13	3	3	34.3
Supinator	Antebrachium	2	9	2	2	41.3
Abductor digiti minimi	Hand	2	12	1	2	56.5
Abductor pollicis brevis	Hand	2	12	1	2	66.0
Adductor pollicis	Hand	2	12	1	2	78.9
Extensor digitorum brevis	Hand	2	7	2	2	53.7
Flexor digiti minimi brevis	Hand	1	6	1	1	68.2
Flexor digitorum brevis	Hand	1	6	1	1	44.5
Flexor pollicis brevis	Hand	1	6	1	1	76.7
Interosseous (Dorsal, 1st)	Hand	2	12	1	2	62.5
Interosseous (Dorsal, 4th)	Hand	1	6	1	1	55.4
Interosseous (Palmar, 1st)	Hand	1	6	1	1	60.6
Interosseous (Palmar, 3rd)	Hand	1	6	1	1	46.9
Lumbrical (1st)	Hand	1	6	1	1	82.7
Lumbrical (4th)	Hand	1	6	1	1	86.3
Lumbricals (undefined)	Hand	1	4	1	1	38.4
Opponens digiti minimi	Hand	1	6	1	1	82.9
Opponens pollicis	Hand	1	6	1	1	74.7
Diaphragm	Trunk	27	171	21	18	25.5

Muscle	Anatomical compartment	No. of data pts	No. of individuals	No. of species	No. of studies	Avg. (%) slow
Erector spinae	Trunk	2	7	2	2	49.9
Extensor caudae lateralis	Trunk	2	10	2	2	38.5
Extensor caudae medialis	Trunk	3	14	3	3	54.2
Flexor caudae lateralis	Trunk	1	3	1	1	53.0
Flexor caudae medialis	Trunk	1	3	1	1	50.0
Hypaxialis lumborum	Trunk	2	7	2	2	52.5
Iliocostalis	Trunk	6	28	6	4	38.8
Iliocostalis (lumbar)	Trunk	6	23	6	3	46.4
Iliocostalis (thoracic)	Trunk	6	20	6	3	40.9
Intercostals	Trunk	4	14	4	3	51.8
Intermamillares	Trunk	1	3	1	1	67.5
mammilloaccessorii L4						
Intermamillares	Trunk	1	3	1	1	81.2
mammilloaccessorii L6						
Interpinales	Trunk	5	17	5	4	29.5
Intertransversarii	Trunk	5	22	5	5	52.8
Longissimus	Trunk	53	411	21	30	29.5
Longissimus capitis	Trunk	1	6	1	1	33.0
Longissimus dorsi	Trunk	36	749	23	16	29.7
Longissimus (lumbar)	Trunk	27	118	21	10	14.8
Longissimus (thoracic)	Trunk	24	133	17	10	21.4
Longissimus (thoracic and lumbar)	Trunk	4	17	2	3	11.6
Longissimus et multifidus (L3)	Trunk	2	38	1	1	21.4
Longissimus et multifidus (Th9)	Trunk	1	21	1	1	73.7
Multifidus	Trunk	16	104	12	14	42.1
Multifidus (lumbar)	Trunk	4	31	4	2	48.3
Multifidus (thoracic)	Trunk	3	10	3	1	42.0
Obliques	Trunk	19	57	19	11	23.7
Panniculus carnosus	Trunk	1	4	1	1	66.2
Quadratus lumborum	Trunk	4	12	4	3	31.4
Rectus abdominis	Trunk	19	57	18	12	22.0
Rotatores	Trunk	3	6	3	2	11.3
Sacrospinalis	Trunk	5	15	5	4	5.6
Semispinalis and longissimus	Trunk	1	3	1	1	46.3
Serratus abdominis	Trunk	9	16	9	2	0.0
Serratus anterior	Trunk	2	7	2	2	52.0
Serratus cervicis	Trunk	1	3	1	1	60.6
Serratus dorsalis	Trunk	1	3	1	1	57.0
Serratus ventralis	Trunk	15	75	10	13	36.6
Spinalis transversospinalis (lumbar)	Trunk	1	3	1	1	30.4
Spinalis transversospinalis (thoracic)	Trunk	2	9	2	2	69.4
Transversus abdominis	Trunk	5	17	5	5	41.8
Capsularis	Pelvic/gluteal	1	3	1	1	99.0
Coccygeus	Pelvic/gluteal	1	1	1	1	3.0
Cremaster	Pelvic/gluteal	1	3	1	1	33.0
Gemelli (undefined)	Pelvic/gluteal	1	3	1	1	69.0

Muscle	Anatomical compartment	No. of data pts	No. of individuals	No. of species	No. of studies	Avg. (%) slow
Gemellus inferior	Pelvic/gluteal	3	10	3	3	34.1
Gemellus superior	Pelvic/gluteal	3	10	3	3	51.3
Gluteofemoralis	Pelvic/gluteal	4	19	4	2	7.6
Gluteals (undefined)	Pelvic/gluteal	4	21	3	3	29.2
Gluteobiceps	Pelvic/gluteal	1	7	1	1	19.8
Gluteus accessorius	Pelvic/gluteal	2	8	2	2	61.2
Gluteus maximus	Pelvic/gluteal	6	36	5	4	34.1
Gluteus maximus ischiofemoralis	Pelvic/gluteal	2	4	2	2	41.1
Gluteus maximus proprius	Pelvic/gluteal	2	4	2	2	19.2
Gluteus medius	Pelvic/gluteal	70	436	35	37	24.7
Gluteus minimus	Pelvic/gluteal	8	50	8	5	40.0
Gluteus profundus	Pelvic/gluteal	2	12	2	2	69.8
Gluteus superficialis	Pelvic/gluteal	8	36	7	6	15.7
Iliacus	Pelvic/gluteal	18	66	18	9	27.6
Iliopsoas	Pelvic/gluteal	2	9	2	2	46.7
Levator ani	Pelvic/gluteal	1	NA	1	1	0.0
Obturator externus	Pelvic/gluteal	5	16	5	5	37.7
Obturator internus	Pelvic/gluteal	4	13	4	4	38.9
Piriformis	Pelvic/gluteal	7	47	7	5	43.5
Psoas major	Pelvic/gluteal	38	189	25	22	23.8
Psoas minor	Pelvic/gluteal	4	39	3	4	33.2
Psoas (major et minor)	Pelvic/gluteal	3	6	3	2	1.0
Quadratus femoris	Pelvic/gluteal	9	52	8	8	77.0
Abductor	Thigh	1	3	1	1	20.0
Adductor brevis	Thigh	15	69	11	9	38.4
Adductor longus	Thigh	16	64	11	11	51.7
Adductor magnus	Thigh	22	83	13	15	22.3
Adductor magnus ischiocondylaris	Thigh	2	4	2	2	48.3
Adductor magnus pubofemoralis	Thigh	2	4	2	2	39.1
Adductor tertius	Thigh	1	6	1	1	1.2
Adductors (undefined)	Thigh	17	56	16	10	10.3
Biceps femoris	Thigh	102	648	46	47	21.0
Biceps femoris (long)	Thigh	3	10	3	3	55.0
Biceps femoris (short)	Thigh	3	10	3	3	40.9
Cruralis	Thigh	1	1	1	1	53.0
Femorococcygeus	Thigh	3	18	3	1	16.5
Gracilis	Thigh	34	152	26	21	17.6
Hamstring (undefined)	Thigh	1	NA	1	1	62.0
Pectineus	Thigh	19	76	16	12	33.2
Quadriceps (undefined)	Thigh	12	138	6	8	18.3
Rectus femoris	Thigh	48	185	35	31	17.7
Sartorius	Thigh	18	77	14	14	37.8
Semimembranosus	Thigh	71	416	38	38	21.2
Semimembranosus accessorius	Thigh	1	1	1	1	99.0
Semitendinosus	Thigh	76	339	45	46	20.4

Muscle	Anatomical compartment	No. of data pts	No. of individuals	No. of species	No. of studies	Avg. (%) slow
Tensor fascia latae	Thigh	16	51	11	14	13.1
Tenuissimus	Thigh	1	1	1	1	20.0
Vastus intermedius	Thigh	49	203	24	30	60.8
Vastus lateralis	Thigh	91	540	56	54	16.4
Vastus medialis	Thigh	45	171	35	28	19.7
Extensor digitorum	Leg	26	169	13	20	15.1
Extensor digitorum lateralis	Leg	3	11	3	3	18.1
Extensor digitorum longus	Leg	59	265	36	32	10.9
Extensor hallucis longus	Leg	11	51	8	8	22.1
Flexor digitorum	Leg	2	10	2	2	31.4
Flexor digitorum fibularis	Leg	6	24	6	4	26.9
Flexor digitorum longus	Leg	13	56	10	8	13.7
Flexor digitorum profundus	Leg	11	22	11	5	19.3
Flexor digitorum superficialis	Leg	10	61	7	9	42.2
Flexor digitorum tibialis	Leg	5	22	5	3	18.2
Flexor hallucis longus	Leg	11	58	9	9	22.1
Gastrocnemius	Leg	71	237	44	32	25.4
Gastrocnemius lateralis	Leg	61	303	43	45	20.1
Gastrocnemius medialis	Leg	66	321	46	47	19.9
Peroneals (undefined)	Leg	2	9	1	1	12.5
Peroneus brevis	Leg	12	50	10	7	21.9
Peroneus longus	Leg	17	72	12	12	23.1
Peroneus tertius	Leg	4	20	4	3	10.0
Plantaris	Leg	49	224	26	34	17.7
Popliteus	Leg	7	21	7	5	31.3
Soleus	Leg	141	820	58	87	69.4
Tibialis anterior	Leg	81	301	46	50	16.8
Tibialis posterior	Leg	15	67	11	11	18.5
Abductor caudae externus	Tail	1	7	1	1	19.8
Abductor caudae internus	Tail	1	7	1	1	16.8
Caudofemoralis	Tail	5	7	4	3	1.4
Flexor caudae brevis	Tail	1	7	1	1	21.6
Flexor caudae longus	Tail	1	7	1	1	27.4
Sacrocaudalis	Tail	2	13	2	2	10.6
Tail	Tail	2	12	1	2	24.5
Tail (distal)	Tail	4	23	3	3	24.9
Tail (mid dorsal)	Tail	1	7	1	1	41.6
Tail (mid ventral)	Tail	1	7	1	1	40.0
Tail (proximal)	Tail	4	23	3	3	11.5
Tail (tip)	Tail	1	7	1	1	5.0
Tail (transitional)	Tail	3	16	3	2	20.3

3.4.13 *Figure 3.9*

Certain skeletal muscles are more represented than others in the literature, with the *m. soleus* being the most represented (**Fig. 3.9**). The *mm. soleus*, *biceps femoris*, *vastus lateralis*, *tibialis anterior*, *semitendinosus*, *semimembranosus*, *gastrocnemius*, *gluteus medius* and *extensor digitorum longus* are the most heavily sampled muscles in terms number of data points (**Fig. 3.9A**). The *mm. soleus*, *longissimus dorsi*, *biceps femoris*, *vastus lateralis*, *gluteus medius*, *semimembranosus*, *longissimus*, *semitendinosus*, *gastrocnemius*, *tibialis anterior*, and *extensor digitorum longus* are the most heavily sampled muscles in terms number of individuals (**Fig. 3.9B**). The *mm. soleus*, *vastus lateralis*, *pectoralis*, *biceps femoris*, *gastrocnemius*, *tibialis anterior*, *semitendinosus*, *deltoid*, *semimembranosus*, and *extensor digitorum longus* are the most heavily sampled muscles in terms number of unique species (**Fig. 3.9C**). The *mm. soleus*, *vastus lateralis*, *tibialis anterior*, *biceps femoris*, *gastrocnemius*, *semitendinosus*, *semimembranosus*, *gluteus medius*, *plantaris*, and *pectoralis* are the most heavily sampled muscles in terms number of studies (**Fig. 3.9D**).

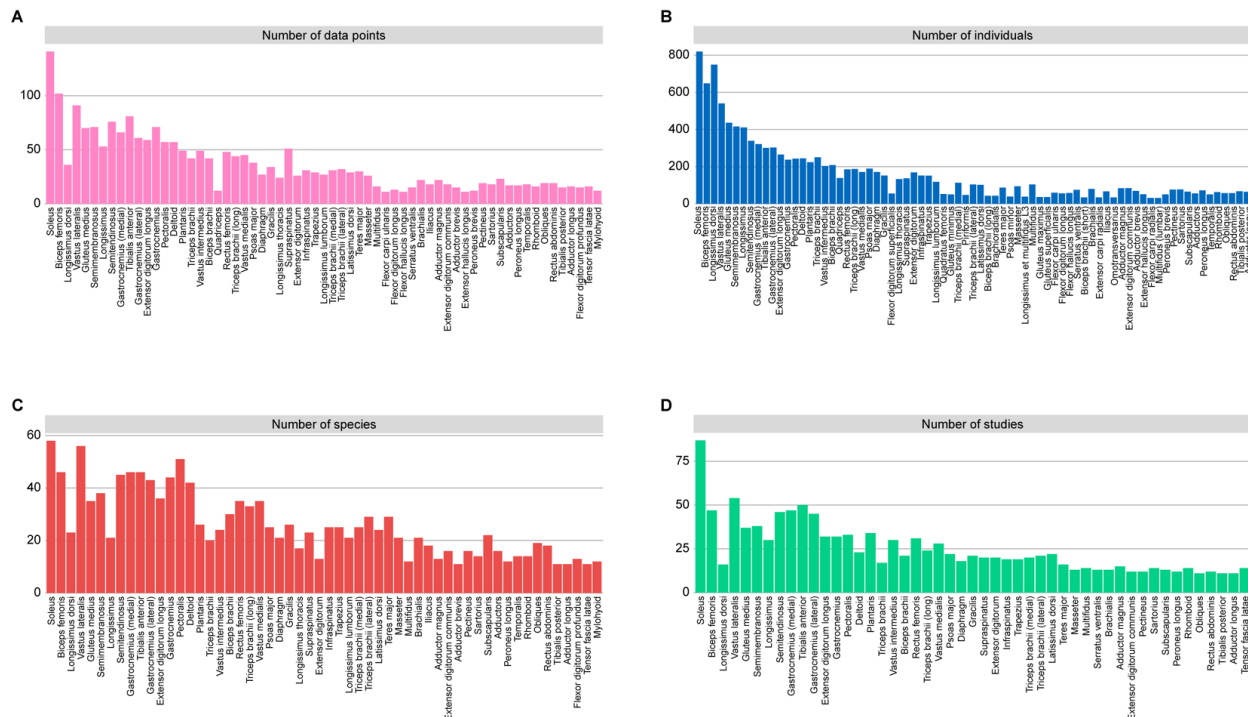
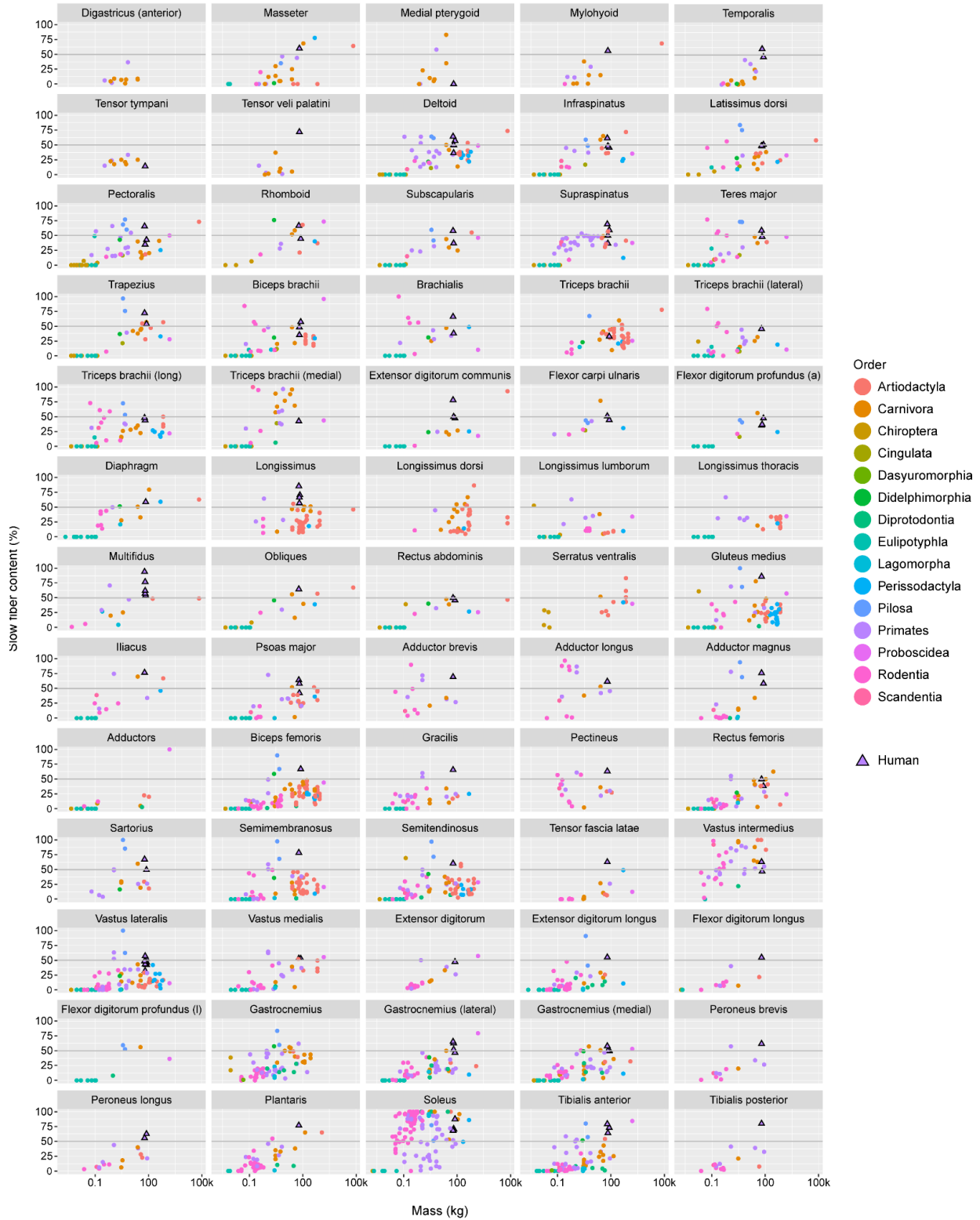


Fig. 3.9. The most represented skeletal muscles in the literature by (a) number of data points, (b) number of individuals sampled across taxa, (c) number of species, and (d) number of studies.

3.4.14 Figure 3.10

The relationship between skeletal muscle slow fiber content (% fibers) and body mass (kg) for muscles that are represented in 10 or more unique species (**Fig. 3.10**).

Fig. 3.10 (next page). The relationship between skeletal muscle slow fiber content (% fibers) and body mass (kg) for muscles that are represented in 10 or more unique species ($n = 65$ muscles). Gray line represents a slow fiber composition of 50%, above which denotes muscles that are slow fiber dominant. Species (data points) are colored by taxonomic order. Humans within the order Primates are distinguished by outlined triangles.



3.5 Experimental design, materials and methods

3.5.1 Eligibility criteria and study selection

Published, peer-reviewed data were compiled for meta-analysis between June 1 2021 and November 30 2022 following a structure similar to Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA)²²⁶, facilitating transparency and ease of reproducibility. Terms relating to mammalian skeletal muscle fiber composition (fiber type slow fast myofiber OR myosin OR "heavy chain" "skeletal muscle" -Xenopus -avian -denervated -transition -develop -developing -stimulated -training -switch -aging -athlete -cardiomyopathy -spaceflight -Duchenne -immobilization -suspension) were queried using academic search systems (Google Scholar, PubMed, and JSTOR) and library databases for relevant primary articles. Reference lists in relevant articles were thoroughly investigated for eligible studies. No restrictions were set on the date of publication.

Articles identified via academic search systems were screened for relevancy by reviewing published abstracts and data tables. When relevant articles were identified, the article DOI and/or citation were recorded and the article PDF and associated supplemental materials were downloaded and renamed under this format: "PublicationDate_FirstAuthorLastName_Species.pdf." If multiple studies from the same first author from the same year exist, the second and third authors' last names were added to the file name.

Relevant articles were then assessed for eligibility. Eligible studies were published in the English language and provided muscle fiber composition data from at least one skeletal muscle from a species belonging to class Mammalia. Studies were not considered eligible for the meta-analysis for the following reasons: (i) muscle fiber composition data were not provided, (ii) muscle fiber composition data provided were ambiguous, (iii) muscle fiber composition data were provided for an unidentified muscle, (iv) muscle fiber composition data were provided for cardiac fibers, (v) muscle fiber composition data were provided for single muscle fibers, (vi) muscle fiber composition data were provided for a mammal under experimental manipulation (e.g., treadmill running, limb immobilization or dietary supplementation regime), (vii) the study duplicated another dataset, (viii) the source was secondary, and (ix) the source was an abstract (**Fig. 3.1**).

3.5.2 Data extraction

The following data were recorded from the text, tables, figures, and supplementary materials of eligible studies when available: sampled species' common name, scientific name, sex, age, breed/strain, method for classification of muscle fiber composition, slow muscle fiber terminology used (e.g., MyHC I, MHC I, Type I, beta, slow oxidative, red or slow twitch), number of individuals sampled, average body mass (kg), muscle(s) sampled and average percent slow fiber content (% fibers).

In most studies, body mass was reported; for those studies that did not report a body mass the species mean was taken from published references^{227,228}. When muscle fiber content was recorded as the percentage of fast muscle fibers within a skeletal muscle, the proportion of slow muscle fibers was derived as 100 minus the total proportion of fast muscle fibers. If slow muscle fiber content was reported from multiple superficial and deep sampling sites across a single muscle, the average across the sampling sites was recorded as the percent slow muscle fiber content of that muscle. Skeletal muscle terms were then assessed for redundancy and updated when necessary to reflect modern anatomical terminology.

Given that most studies were published between 1965 and 1999, binomial and common names were updated when necessary to reflect the current understanding of phylogenetic relationships. Taxonomic data (i.e., class, infraclass, magnorder, superorder, order, suborder, infraorder, parvorder, clade, superfamily, family, subfamily, tribe, and subspecies) provided by NCBI Taxonomy²²⁹ was recorded for each species when available. Species were then categorized into one of four locomotor types based on how the species navigates through its habitat matrix: *arboreal*, *marine*, *terrestrial*, and *volant* (**Fig. 3.8**).

3.6 Bridge

Chapter 3 provides broader context for the mammalian myofiber dataset generated from the meta-analysis in Chapter 2, identifying avenues for further interspecific comparisons, and highlighting the need for histochemical data from underrepresented species and muscles. Given the genetic basis of muscle myofiber composition, Chapter 4 then turns to identifying the genetic mechanisms that regulate myofiber type as a first step toward understanding human muscle evolution.

4 CIS-REGULATORY ELEMENTS SHAPE MYOGENESIS AND MYOFIBER DIFFERENTIATION IN LATE EMBRYONIC AND ADULT MOUSE MUSCLES

This work was submitted for publication in *Scientific Reports* for their special issue on Myogenesis in June 2025. Samantha R. Queeno (SRQ), Terence D. Capellini (TDC), and Kirstin N. Sterner (KNS) conceptualized the study; KNS and TDC supervised the project; Alexander S. Okamoto and SRQ performed the experiments; SRQ wrote the manuscript, analyzed the data, and prepared the figures; Matthew C. O'Neill and Damien M. Callahan substantially revised the manuscript for intellectual content, and all authors edited the manuscript. All authors approved the final version of the manuscript.

4.1 Introduction

Skeletal muscle accounts for a substantial portion of mammalian body mass^{202,428} and plays a fundamental role in locomotion^{111,118,424}, thermoregulation¹¹³ and nutrient storage^{114,115}. Each muscle contains a heterogeneous population of multinucleated muscle cells known as myofibers, which collectively contribute to muscle function^{135–137,429}. Myofibers can be classified into distinct types based on a range of criteria, including physiological properties^{130–132,139,141}, protein content^{135,136}, developmental timing during myogenesis¹³³, and the myosin heavy chain (MyHC) isoforms expressed within thick filaments^{126,127}. Because myofiber physiology and contractile function are heavily influenced by MyHC isoform expression, MyHC isoforms, and the genes that encode them, are commonly used as molecular markers of myofiber type^{128,129}.

Within a single muscle, the expression of MyHC genes and isoforms varies across developmental stages and between myofiber types. In mammals, developing myofibers first express embryonic MyHC-emb (*Myh3*), followed by neonatal MyHC-neo (*Myh8*)¹³⁹. Shortly before birth, the expression of these developmental isoforms declines in favor of the MyHC isoforms that distinguish mature myofiber types, largely driven by muscle innervation^{133,223,224,430–432}. After birth, mature myofibers are classified as either slow (i.e., slow-twitch or type-I) or fast (i.e., fast-twitch or type-II), based on the distinct contractile kinetics in the motor domain (S1 region) of MyHC's globular head⁴³³. Slow-contracting myofibers predominantly express slow-oxidative MyHC-I/β (*Myh7*), whereas fast-contracting myofibers primarily express a range of MyHC-II subtypes, including MyHC-IIa (*Myh2*), MyHC-IIx/d (*Myh1*), and MyHC-IIb (*Myh4*). Slow myofibers are highly fatigue-resistant, partially due to slower motor domain kinetics that reduce the rate of ATP hydrolysis, and a metabolic preference for synthesizing ATP via oxidative phosphorylation^{131,138–141}. In contrast, fast myofiber generate velocity and power (force x velocity) several-fold greater than slow myofibers. Their high rate of ATP hydrolysis and tendency to rely on

glycolysis to synthesize ATP create a metabolic environment rich in inorganic phosphate and protons, making them more susceptible to fatigue^{143–146}.

Individual muscles contain a mix of fast and slow myofibers, and variation in this composition can impact the contractile and metabolic properties of both muscle bundles and whole tissue^{150,151}, ultimately affecting locomotor performance. Across mammals, muscle myofiber composition varies in relation to body size and locomotor strategy^{423,424}. Humans, sloths and slow lorises are unusual among terrestrial mammals in possessing muscles with a high proportion of fatigue-resistant slow myofibers (‘slow-biased’ muscles), which support long-duration locomotor behaviors such as long-distance walking and running, extended tree hanging, and slow climbing^{248,423,424,434}. In contrast, the muscles of mice and other small mammals are dominated by fast myofibers (‘fast-biased’ muscles), which enable higher-powered, shorter-duration locomotor behaviors such as sprinting and frequent bursts of movement. Experimentally altering the physiological properties of muscle tissue in mice has been shown to improve muscle fatigue-resistance (i.e., endurance) and average daily travel distance¹⁵², although factors like oxygen transport also influence endurance capacity⁷.

Underlying shifts in muscle myofiber content between muscles, and more broadly, between species, are modifications to developmental gene regulation. Mutations in *cis*-regulatory elements (CREs), such as enhancers and silencers, can alter patterns of gene expression at key developmental stages in a tissue-specific manner, leading to phenotypic variation and evolutionary divergence between species^{174–184,192,193}. Therefore, evolutionary selection acting on developmental CREs that regulate the expression of genes influencing muscle myofiber content may be critical to understanding the physiological differences between myofiber types in a body, and the same muscle between species⁴²³. While MyHC ‘super-enhancers’ that regulate the expression of genes within the fast and slow MyHC gene clusters have recently been identified^{195,196}, CREs that control the expression of other genes important to the determination of myofiber physiology, such as those involved in mitochondrial biogenesis or MyHC signaling cascades, are less understood. Identifying such elements would provide deeper insight into muscle development and biology and offer valuable targets for investigating species-specific differences in muscle myofiber composition, such as those observed between humans and African apes^{423,424}.

Here, we used a mouse model to characterize the regulatory landscape of individual limb muscles that differ in slow myofiber content at a late embryonic (E18.5) and an adult time point, to identify CREs that may regulate genes influencing myofiber type and muscle physiology. Overall, patterns of gene expression and chromatin accessibility reflect differences due to time point, with limb patterning driving variation in E18.5 muscles and myofiber physiology driving variation in adult muscles. By targeting non-coding regions with greater specificity to muscle tissue, we identified nine enhancers and three silencers that may regulate genes contributing to myofiber type and adult muscle function. Our results highlight the

importance of spatiotemporal gene regulation during myogenesis and suggest that muscle-specific CREs controlling genes involved in myofiber metabolism and contractile function are promising targets for investigating species-specific differences in muscle myofiber composition.

4.2 Methods

4.2.1 Mice

All mouse procedures were performed in accordance with relevant guidelines, regulations and protocols approved by the Harvard University and University of Oregon Institutional Animal Care and Use Committees (Capellini protocol: 13-04-161-2; Sterner protocol: AUP-20-04). All mice were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*⁴³⁵ at the Harvard University Biological Research Infrastructure Mouse Facility (Capellini laboratory mouse room). This study is reported in accordance with ARRIVE guidelines⁴³⁶.

Adult FVB/NJ mice were obtained at 3-4 months of age. Mice were free of disease or injury at the time of sample collection and were not previously involved in biological research. Male and female mice were used to establish timed matings, and at embryonic stage (e) 18.5, a stage relevant to our studies^{133,437}, pregnant females were euthanized to acquire embryos. Adult mice were exposed to CO₂ at a constant rate for 5 minutes and secondary euthanasia was performed via cervical dislocation by a trained investigator. E18.5 embryos were dissected under a microscope in 1X PBS on ice, decapitated to ensure rapid euthanasia, and anatomically sexed⁴³⁸. To minimize variation attributed to X-chromosome silencing factors (e.g., *Xist*, *Tsix*), only muscle samples from male mice were used for ATAC-seq and RNA-seq analysis. Four hind limb muscles were chosen for dissection based on previously described differences in slow myofiber content¹⁵⁶. The soleus and vastus intermedius represent slow-biased muscles (58% and 45% slow, respectively), and the gastrocnemius and vastus lateralis represent fast-biased muscles (8% and 0% slow, respectively).

4.2.2 RNA-seq

Soleus, gastrocnemius, vastus intermedius, and vastus lateralis samples were collected from adult male ($n = 6$) and male E18.5 mice ($n = 6$) and preserved in TRIzol reagent (Invitrogen). Muscles were identified using a reference⁴³⁹. To allow direct access to the target muscles, the skin and any superficial muscles (such as the biceps femoris, caudofemoralis, semimembranosus, and semitendinosus) were removed using sharp dissection scissors and forceps. After collection, muscle samples were pulverized using an electric homogenizer and subjected to phenol-chloroform extraction. Total RNA was isolated and purified using a Zymo Direct-zol RNA Microprep Kit following the manufacturer's instructions.

RNA was eluted in nuclease-free water and evaluated for quantity and quality. RNA concentrations were measured using a Qubit fluorometer, RNA purity was assessed using a NanoDrop spectrophotometer, and RNA integrity was assessed using an Agilent TapeStation. Only samples with RNA Integrity Number (RIN) scores above 7.0 and matched across all four muscles from the same individual were retained for library preparation and sequencing.

RNA-seq libraries were prepared by the Harvard Bauer Core Facility (HBCF) using the Kapa mRNA HyperPrep Kit and automated on a PerkinElmer Sciclone NGS Workstation. RNA was reverse transcribed into cDNA using random primers. Total RNA concentration was measured using the Quant-iT RiboGreen RNA Assay Kit, and total RNA integrity was assessed using an Agilent BioAnalyzer 2100 RNA Nano Kit. Libraries were pooled and assessed for quality using qPCR. Deep sequencing was performed on an Illumina NovaSeq6000 platform using 2 x 100 bp paired-end reads, targeting a minimum depth of 30 million reads per sample⁴⁴⁰. RNA-seq sample details, quality control metrics, and read processing information are provided in **Supplementary Excel Table 4.1**.

Demultiplexed sequencing data were processed using the University of Oregon's high-performance computing cluster, Talapas, and analyzed in RStudio⁴⁴¹ using R (v.4.5.0)⁴⁴² following the Encyclopedia of DNA Elements (ENCODE) RNA-seq guidelines (www.encodeproject.org/about/experiment-guidelines/). Sequence quality, GC content, and adapter contamination was assessed using FastQC (v.0.11.5)⁴⁴³. Adapter sequences and low-quality bases (Phred score < Q20) were trimmed using Trimmomatic (v.0.39.0)⁴⁴⁴. To enrich for mRNA and reduce background noise, residual ribosomal RNA reads were removed using RiboDetector (v.0.3.1)⁴⁴⁵. Processed reads were aligned to the mouse reference genome (mm10) using STAR (v.2.7.11)⁴⁴⁶.

To assess sample relationships, Principal Component Analysis (PCA) was performed using the plotPCA function in R, and Multidimensional Scaling (MDS) analysis was performed using the R package PoiClaClu (v.1.0.2.1)⁴⁴⁷ (**Appendix D**). One adult vastus intermedius sample ("A Vast Int 7/27/21 RNA") was excluded from downstream analysis due to clustering anomalies. Therefore, $n = 5$ adult vastus intermedius samples and $n = 47$ RNA-seq samples in total.

4.2.3 Analysis of differentially expressed genes (DEGs)

To quantitatively compare gene expression (relative read count) across conditions, a raw read count matrix was generated from processed RNA-seq data using the featureCounts function from Subread (v.2.0.6)⁴⁴⁸. Differential expression analysis was performed using the R package DESeq2 (v.1.44.0)⁴⁴⁹ to identify genes differentially expressed across time points (E18.5 vs. adult), myofiber biases (fast vs. slow), anatomical regions (calf vs. thigh), and muscle identities (soleus vs. gastrocnemius vs. vastus intermedius vs. vastus lateralis), as well as genes differentially expressed within each time point (E18.5

Fast vs. E18.5 Slow, Adult Calf vs. Adult Thigh), using a false discovery rate (FDR)⁶³ threshold of < 0.05. Genes with fewer than five read counts across all samples were excluded, leaving 20,889 genes for analysis. Genes were considered significantly differentially expressed (DEGs) if they met the thresholds of $|\log_2 \text{fold change}| > 0.6$ and adjusted $p < 0.05$. For visualizations, library-normalized counts were transformed based on the mean-variance relationship across genes using the variance-stabilized transformation function `vst()` from DESeq2 to account for differences in variance. Visualizations were created using the R package `ggplot2` (v.3.5.1)⁴⁵⁰.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using the R package `clusterProfiler` (v.4.12.3)⁴⁵¹. The `simplify` function was applied to reduce redundancy among enriched GO terms. Gene set enrichment analysis (GSEA) was performed using the R package Fast Gene Set Enrichment Analysis (FGSEA, v.1.30.0)⁴⁵². Human orthologs were retrieved using the `mouse2human` function from the R package `HomoloGene` (v.1.4.68.19.3.27), and human Disease Ontology (DO) enrichment analysis was performed using the R package Disease Ontology Semantic and Enrichment Analysis (DOSE, v.3.30.5)⁴⁵³. To reduce redundancy in DO enrichment results, a semantic similarity matrix was computed using the `doSim` function followed by hierarchical clustering. The resulting dendrogram was cut at a height of 0.3 to define semantically similar clusters, from which the term with the lowest adjusted p-value was retained. All statistical analyses applied a q-value threshold of < 0.05.

4.2.4 ATAC-seq

Additional samples of the soleus, gastrocnemius, vastus intermedius, and vastus lateralis were collected from adult male ($n = 3$) and male E18.5 mice ($n = 3$ pooled litters). To ensure a minimum of 50,000 cells per embryonic muscle sample for ATAC-seq analysis^{198,199}, each E18.5 biological replicate consisted of pooled muscles (both left and right sides) from up to five male embryos from the same litter. Muscle nuclei were isolated from undigested tissue using a previously established protocol⁴⁵⁴. Cell viability was assessed using trypan blue exclusion and quantified with a hemocytometer under an inverted microscope. Only samples with <10% cell death were subjected to an optimized ATAC-seq protocol⁴⁵⁵. Briefly, muscle nuclei were lysed to release DNA and treated with Tn5 transposase to tag transcriptionally accessible fragments of DNA not bound to histones (i.e., potential non-coding regulators of gene expression such as CREs). Transposed DNA was purified using the Zymo DNA Clean and Concentrator Kit following the manufacturer's instructions. DNA fragments were then barcoded with indexed primers and PCR-amplified to generate ATAC-seq libraries. Libraries were size-selected using the OMEGA Bead Purification Protocol, and concentrations were determined using the KAPA Library Quantification Complete Kit.

ATAC-seq library quality was assessed by the HBCF using the Agilent Bioanalyzer 2100 DNA High Sensitivity Kit and the Agilent TapeStation 4200 (D1000 ScreenTape assay). Libraries were pooled and evaluated for quality using qPCR and the Illumina MiSeq Nano system. Library pools were adjusted based on the Illumina MiSeq Nano results to optimize distribution and final loading concentration. Deep sequencing was performed on an Illumina NovaSeq6000 platform using 2 x 100 bp paired-end reads, targeting a minimum depth of 50 million reads per sample⁴⁴⁰. ATAC-seq sample details, quality control metrics, and primer sequences are provided in **Supplementary Excel Table 4.2**.

Demultiplexed sequencing data were processed using the University of Oregon's high-performance computing cluster, Talapas, and analyzed in RStudio⁴⁴¹ using R (v.4.5.0)⁴⁴² following ENCODE ATAC-seq guidelines (www.encodeproject.org/about/experiment-guidelines/). Sequence quality, GC content, and adapter contamination was assessed using FastQC (v.0.11.5)⁴⁴³. Adapter sequences and low-quality bases (Phred score < Q20) were trimmed using Trimmomatic (v.0.39.0)⁴⁴⁴, and reads were aligned to the mm10 genome using Bowtie2 (v.2.4.4)⁴⁵⁶. To target more high-value portions of the genome, reads mapping to the mitochondrial genome were removed using SAMtools (v.1.14.0)⁴⁵⁷, and duplicate reads were removed using Picard (<http://broadinstitute.github.io/picard>). To evaluate variation in accessibility across samples, an analysis of variance (ANOVA) with Tukey's post hoc test and a Kruskal-Wallis test were performed. Statistical significance was defined as $p < 0.05$.

4.2.5 *Analysis of differentially accessible peaks of open chromatin*

Filtered BAM files were assessed for read alignment enrichment (i.e., peaks) using MACS2 (v.2.1.1)⁴⁵⁸ with the callpeak parameters: -B -f BAMPE -g mm -p 0.05. Resulting MACS2 peak output files were merged into a non-redundant consensus peak set yielding 928,789 peaks. A matrix of peak accessibility across samples was generated using the countOverlaps function from the R package GenomicRanges (v.1.60.1)⁴⁵⁹. Differentially accessible regions were identified using DESeq2 to compare peak accessibility between time points (E18.5 vs. adult), myofiber biases (fast vs. slow), and anatomical regions (calf vs. thigh), applying an FDR threshold of < 0.05. Peaks accessible in fewer than three samples were excluded, leaving 252,701 open chromatin regions for analysis. For visualizations, peak counts were then transformed using VST as previously described.

Reproducible peaks across biological replicates were identified using an irreproducible discovery rate (IDR)⁴⁶⁰ threshold of < 0.05. Differences in peak count and mitochondrial read enrichment were assessed using ANOVA with Tukey's post hoc test, Kruskal-Wallis test, and paired *t*-test, with statistical significance defined as $p < 0.05$. Overlap between peak sets was evaluated using pairwise Jaccard index analysis.

To reduce background noise and exclude peaks likely representing pleiotropic CREs active across

multiple tissue types⁴⁶¹, IDR peaks overlapping mm10 blacklist regions⁴⁶² and an IDR peak set generated from embryonic mouse brain tissue⁴⁶³ were removed using BEDTools (v.2.25.0)⁴⁶⁴. Although some open chromatin peaks shared between brain and muscle may contribute to muscle biology, this conservative filtering approach produced ‘brain-filtered’ IDR peak sets predicted to contain CREs with increased specificity to muscle tissue.

To examine inter-species conservation, phyloP scores for 60 mammalian species (phyloP60ways)⁴⁶⁵ were obtained from the University of California Santa Cruz (UCSC) Genome Browser (<http://genome.ucsc.edu>)⁴⁶⁶ and used to extract per-base conservation scores for IDR peak sets before and after brain-filtering. To control for variation in peak size, all peaks were resized to a fixed width of 400 bp, centered at ± 200 bp from each peak midpoint. Per-base conservation scores were averaged across each fixed-width peak and mean peak conservation scores were then averaged across each IDR peak set to generate an overall peak set conservation score. Conservation scores before and after brain-filtering were compared using a paired *t*-test and an ANOVA with Tukey’s post hoc test. See **Appendix E** for MACS2 and IDR peak counts, as well as mean per-base conservation scores. Unfiltered and brain-filtered IDR peak sets were annotated to genomic features using the R package ChIPseeker (v.1.36.0)⁴⁶⁷, and significant differences in genomic feature representation were assessed using a G-test and an ANOVA with Tukey’s post hoc test.

Brain-filtered IDR peak sets were intersected using the multiinter function from BEDTools to identify regions reliably called as peaks across multiple samples. This generated nine non-overlapping open chromatin peak sets, referred to as ‘condition-specific’ peak sets. The E18.5 condition-specific peak set includes peaks found in all four muscles (soleus, gastrocnemius, vastus intermedius and vastus lateralis) at the E18.5 time point but not at the adult time point. Conversely, the Adult condition-specific peak set includes peaks present across all four muscles at the adult time point but not at E18.5. Several condition-specific peak sets further distinguish open chromatin accessibility by time point and myofiber bias. The E18.5 Fast and E18.5 Slow condition-specific peak sets contain peaks found exclusively in embryonic muscles, but specific to fast-biased muscles (gastrocnemius and vastus lateralis) or slow-biased muscles (soleus and vastus intermedius), respectively. Similarly, the Adult Fast and Adult Slow condition-specific peak sets contain peaks found exclusively in adult muscles, but specific to fast-biased or slow-biased muscles, respectively. The Fast condition-specific peak set includes peaks found exclusively across fast-biased muscles regardless of time point; the Slow condition-specific peak set similarly includes peaks found exclusively across slow-biased muscles regardless of time point. The Universal condition-specific peak set includes peaks accessible across all muscles at both time points. Significant differences in condition-specific peak set enrichment were compared using a negative binomial regression.

Condition-specific peak sets (i.e., Adult, Adult Fast, Adult Slow, E18.5, E18.5 Fast, E18.5 Slow, Universal, Fast, Slow) were annotated to genomic features using ChIPseeker and significant differences in feature representation were compared as described previously. Each condition-specific peak set was also annotated to nearby genes and analyzed for functional enrichment using the Genomic Regions Enrichment of Annotations Tool (GREAT, v.4.0.4)⁴⁶⁸. Transcription factor motif enrichment was assessed using Hypergeometric Optimization of Motif EnRichment (HOMER, v. 4.10.4)⁴⁶⁹ on 400 bp windows centered $\pm$ 200 bp from the midpoint of each peak. The following Homer findMotifsGenome.pl parameters were used: -size given -mask. To evaluate inter-species conservation, phyloP scores were obtained from the UCSC Genome Browser and used to extract per-base conservation scores for each fixed-size (400 bp) condition-specific peak set, as described above. Average conservation scores for each condition-specific peak set were then compared to a randomly generated background using a Wilcoxon Rank-Sum test.

To visually assess the epigenetic landscape surrounding peak sets, MACS2 narrowPeak and condition-specific peak set BED files were imported into the UCSC Genome Browser. The following annotation tracks were loaded: Base Position, GENCODE VM23, NCBI RefSeq, ENCODE cCREs, ORegAnno, ENCODE regulation, CpG Islands, JASPAR Transcription Factors, FANTOM5, RefSeq Func Elems, ReMap ChIP-seq, Placental Mammal Basewise Conservation by PhyloP (PlacentalCons), and RepeatMasker. To target regions that may function as CREs, additional mouse muscle ChIP-seq datasets^{195,470–472} and MyHC gene super-enhancer annotations^{196,473} were included as custom tracks. An independent ATAC-seq dataset (GEO SuperSeries accession GSE123879)⁴⁷² was processed using our peak-calling pipeline and imported into the UCSC Genome Browser to compare open chromatin signals across a wider range of muscles, strains, and library preparation methods.

4.2.6 Luciferase reporter vector construction

Candidate CREs were prioritized based on multiple criteria, including chromatin accessibility patterns across ATAC-seq datasets, strength of regulatory signal from UCSC Genome Browser tracks (e.g., ENCODE CREs) and mouse ChIP-seq datasets^{195,470–472}, mammalian conservation scores (phyloP60ways), nearby gene function, and nearby gene expression patterns derived from RNA-seq. High priority was given to candidates exhibiting strong regulatory signals and high evolutionary conservation located within $\pm$ 100 kb of DEGs known to influence fast and/or slow myofiber phenotypes. Of particular interest were candidates accessible exclusively in slow-biased peak sets and located near slow myofiber genes up-regulated in slow-biased muscles, as well as candidates accessible only in fast-biased peak sets and located near fast myofiber genes up-regulated in fast-biased muscles (see example in **Appendix F**).

Candidate DNA sequences were narrowed to < 500 bp regions based on phyloP conservation scores

and screened for restriction enzyme cut sites using NEBcutter (v.3.0.17)⁴⁷⁴. Twelve candidate CRE sequences were cloned upstream of the firefly luciferase reporter gene (*luc2*) promoter in the pGL4.23 [luc2/minP] vector (E8411, Promega) by Azenta Life Sciences. All reporter vector constructs were verified by Sanger sequencing⁴⁷⁵. Candidate CRE sequences, nearby gene functions, conservation scores, and restriction enzyme cut sites are provided in **Supplementary Excel Table 4.3**.

4.2.7 Cell culture

Mouse C2C12 myoblasts (ATCC, CRL-1772) were cultured in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, on 0.1% gelatin-coated cultureware at 37 °C in a humidified atmosphere with 5% CO₂. Growth medium was replaced every 2–3 days, and myoblasts were subcultured when reaching ~80% confluency. For transfection experiments, undifferentiated C2C12 myoblasts were seeded into 0.1% gelatin-coated 48-well plates and cultured for 24–48 hours prior to transfection.

4.2.8 Transfection and luciferase reporter assays

C2C12 myoblasts were transfected in 48-well plates using Lipofectamine 3000 (Invitrogen) following the manufacturer's protocol. Three independent transfection experiments ($n = 3$) were conducted, each with at least six technical replicates per reporter vector construct. An empty pGL4.23 vector (E8411, Promega) was used to determine baseline *luc2* expression. All reporter vector constructs were co-transfected with a pGL4.74 [hRluc/TK] *Renilla* vector (E6921, Promega) to control for transfection efficiency. After optimization, cells were harvested 48 hours post-transfection and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (E1910, Promega) on a GloMax Navigator microplate luminometer (GM2010, Promega), following the manufacturer's instructions. Luciferase activity was calculated as the ratio of firefly (*luc2*) to *Renilla* (*Rluc*) luminescence, averaged across the technical replicates. The mean *luc2/Rluc* ratio for each reporter vector construct was then normalized to the empty pGL4.23 control vector to confirm regulatory activity in C2C12 myoblasts. Significant differences in normalized luciferase activity were assessed using an unpaired two-tailed Student's *t*-test, and Fisher's combined probability test was used to integrate p-values across the three transfection experiments. Data are presented as mean $\pm$ SEM (standard error of the mean) and statistical significance is indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

4.3 Results

4.3.1 Time point-dependent gene regulation shapes gene expression and chromatin accessibility patterns in mouse hind limb muscles.

Given the dynamic nature of myogenesis and the distinct physiological properties of adult myofiber types, we hypothesized that differences in gene expression and chromatin accessibility would be largely driven by time point. To test this, we generated gene expression (RNA-seq) and chromatin accessibility (ATAC-seq) profiles for late embryonic (E18.5) and adult muscles (see Methods, **Fig. 4.1a**) and assessed sample similarity using PCA. Both datasets separated by time point along PC1 (**Fig. 4.1b,c**), and by adult myofiber bias along PC2 (**Fig. 4.1d,e**). Interestingly, RNA-seq data clustered by anatomical region at E18.5 (**Fig. 4.1f**) and by muscle identity at the adult time point (**Fig. 4.1g**). These patterns were not observed in the ATAC-seq data, likely due to low peak overlap across samples (Jaccard index < 0.3; **Appendix G**, as expected for this data type⁴⁷⁶).

We next performed differential expression analysis to identify genes significantly up-regulated at each time point. We identified 11,822 DEGs, including 6,589 genes up-regulated in E18.5 muscles and 5,233 up-regulated in adult muscles (**Fig. 4.1h**). Notably, embryonic *Myh3* and neonatal *Myh8* were among the top 15 DEGs in embryonic muscles, while MyHC-IIb *Myh4* ranked among the top 15 in adult muscles. Genes up-regulated at E18.5 were enriched for GO terms and KEGG pathways relating to limb development (*Actn1*, *Hoxd13*, *Myf5*, *Myl4*, *Myl6b*, *Myo1c*, *Myh10*, *Myo15b*, *Tnnt2*, *Tpm4*), while genes up-regulated in adult muscles were associated with muscle metabolism (*Adipoq*, *Foxo1*, *mt-Atp8*, *Ppargc1a*, *Pparg*) and myofiber differentiation (*Actn3*, *Mybpc2*, *Myh2*, *Myh7*, *Myoz1*, *Tnncl*, *Tnni2*, *Tnnt1*, *Tnnt3*, *Tpm3*) (**Supplementary Excel Table 4.4**). Together, these findings indicate that patterns of gene expression and chromatin accessibility largely reflect the time-dependent regulation of genes essential for limb development and adult muscle physiology.

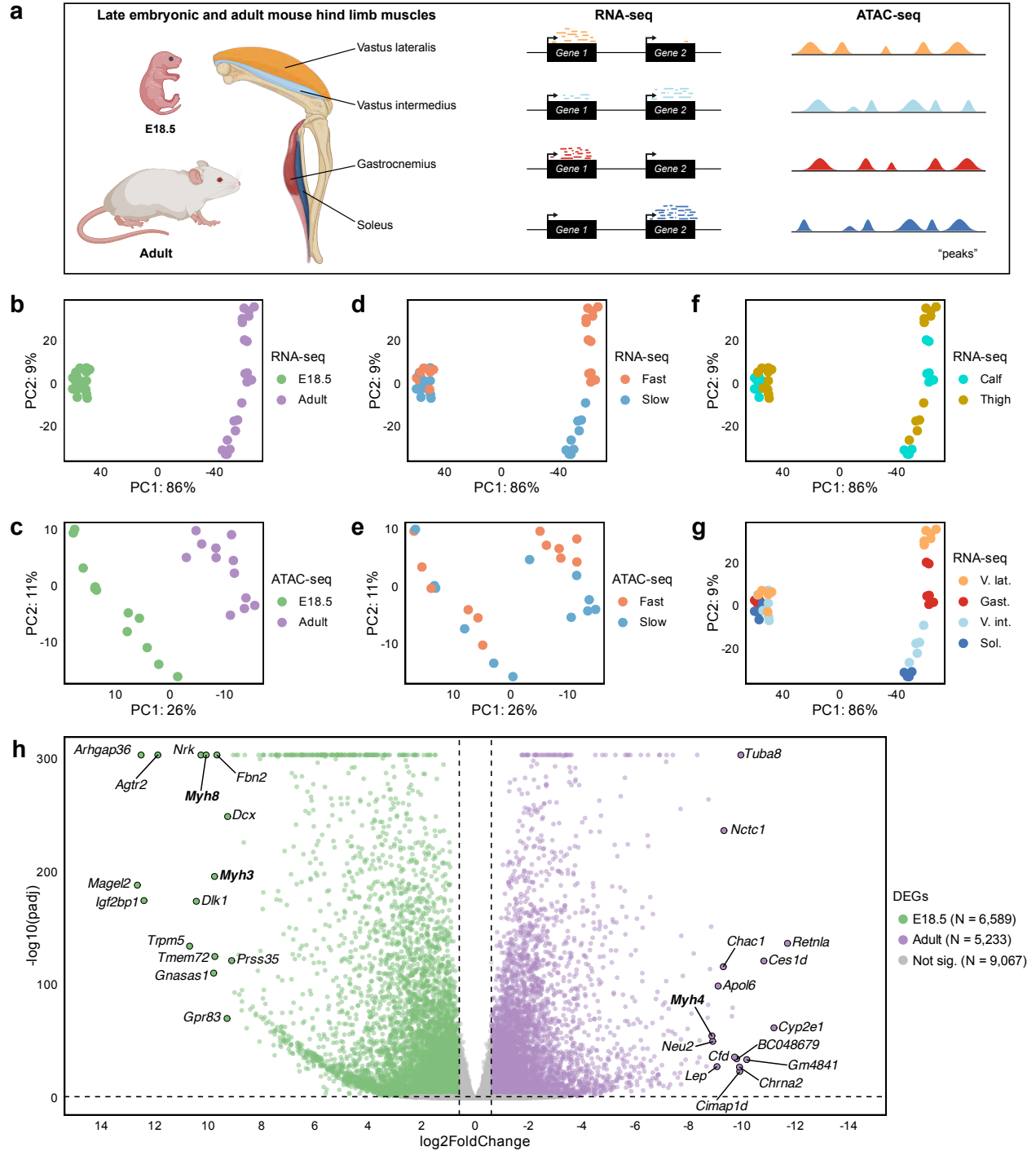
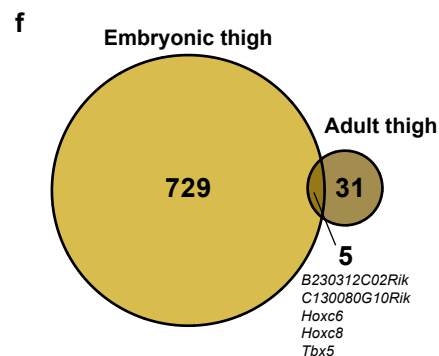
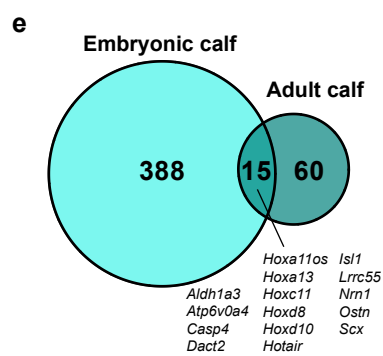
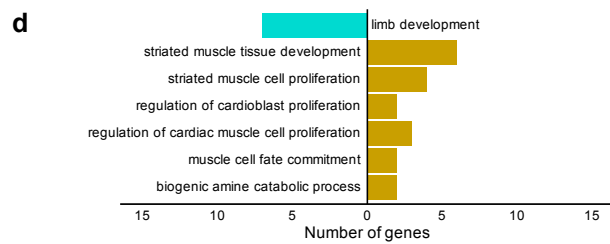
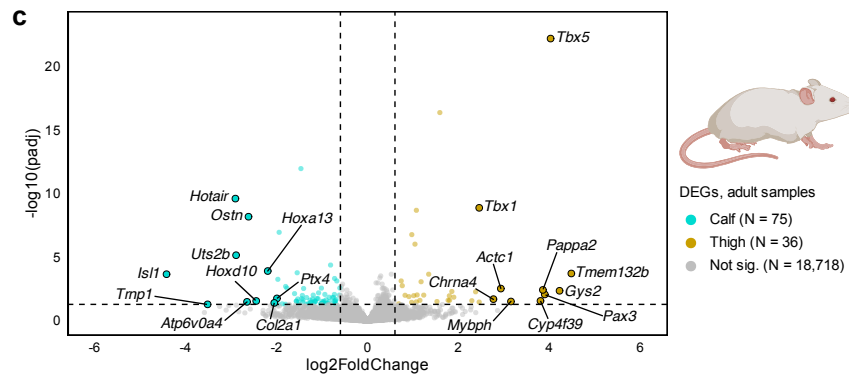
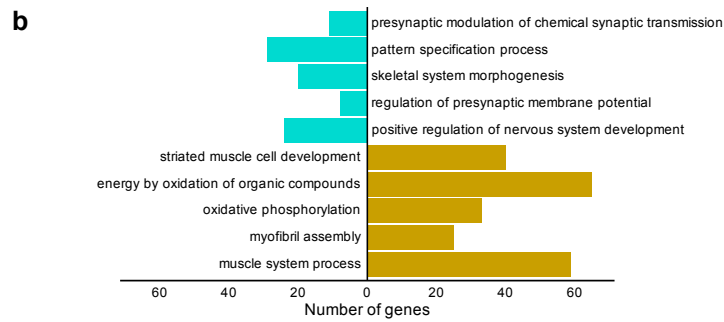
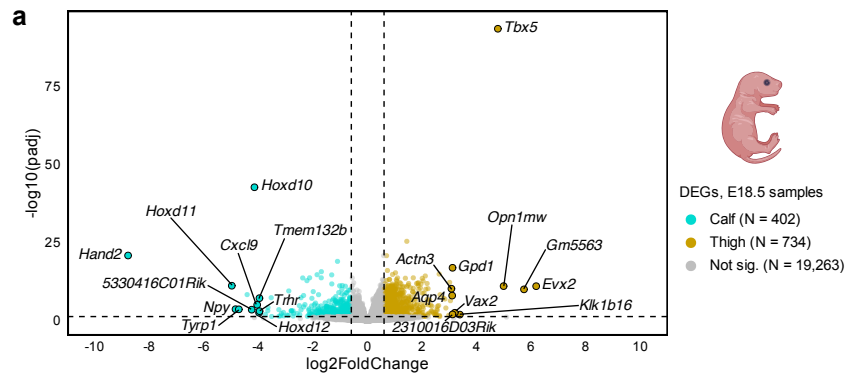


Fig. 4.1. Gene expression and chromatin accessibility in mouse hind limb muscles at a late embryonic (E18.5) and adult time point. (a) Experimental schematic. (b-g) PCA of RNA-seq and ATAC-seq data. PCA of normalized transcriptome data is colored by (b) time point, (d) myofiber bias, (f) anatomical region, and (g) muscle identity ($n = 47$). (c,e) PCA of MACS2-called ATAC-seq peaks colored by (c) time point and (e) myofiber bias ($n = 24$). (h) Volcano plot showing DEGs between E18.5 (green) and adult (purple) muscles. Gray dots represent genes that did not meet the significance threshold ($|\log_2 \text{fold change}| \geq 0.6$; adjusted $p \leq 0.05$). Gene symbols indicate the top 15 DEGs for each time point. Bold gene symbols highlight significantly differentially expressed MyHC genes.

4.3.2 E18.5 transcriptomes suggest earlier differentiation of thigh musculature.

To identify genes driving the time point-dependent clustering of RNA-seq data by anatomical region, we performed differential expression analysis between calf and thigh muscles at each time point and assessed the biological functions of DEGs using GO enrichment analysis. As expected, more genes were differentially expressed between calf and thigh muscles at E18.5 ($n = 1,136$; **Fig. 4.2a**) than at the adult time point ($n = 111$; **Fig. 4.2c**). At E18.5, more genes were up-regulated in thigh muscles ($n = 734$) than calf muscles ($n = 402$), possibly reflecting the earlier regionalization of thigh musculature⁴⁷⁷. Notably, genes involved in distal/posterior limb patterning (*Bmp7*, *Eya2*, *Hoxa11*, *Hoxa13*) were up-regulated in E18.5 calf muscles, whereas genes associated with adult fast myofibers (*Actn3*, *Myh2*, *Myh4*, *Tnnc2*, *Tnni2*, *Tnnt3*) were up-regulated in E18.5 thigh muscles, suggesting that more anterior and proximal muscle groups (e.g., thigh) may differentiate earlier^{478,479}. Indeed, genes up-regulated in E18.5 thigh muscles were enriched for terms related to myofiber development, while those up-regulated in E18.5 calf muscles were enriched for pattern specification (**Fig. 4.2b**). Similar trends were observed in KEGG pathway and gene set enrichment analyses (**Supplementary Excel Table 4.4**). At the adult time point, genes up-regulated in calf muscles were again associated with limb patterning (*Hoxa13*, *Hoxc11*, *Hoxd10*, *Hotair*), while those up-regulated in thigh muscles were linked to muscle cell proliferation (*Tbx1*, *Tbx5*, *Ephb1*, *Mapk11*) (**Fig. 4.2d**). Only a small subset of DEGs identified at E18.5 remained up-regulated in adult muscles. Genes up-regulated in calf muscles across time points ($n = 15$; **Fig. 4.2e**) were associated with limb patterning^{478,480,481}, muscle development^{482–485}, and the central nervous system^{486–489}. Those up-regulated in thigh muscles across time points ($n = 5$, **Fig. 4.2f**) were largely involved in regional specification^{490–492}. These findings suggest that limb patterning is a primary driver of gene expression variation at E18.5, with thigh musculature undergoing earlier differentiation than calf musculature.

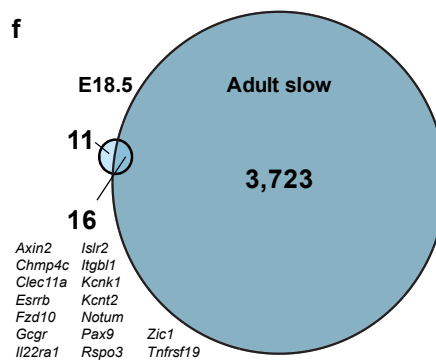
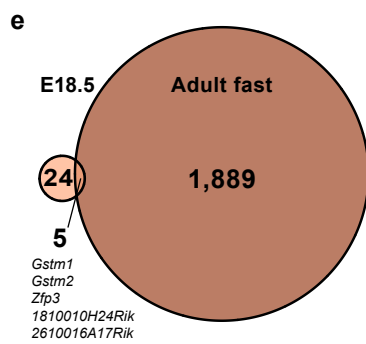
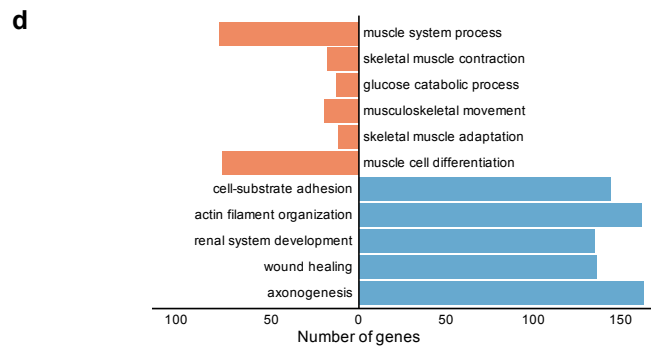
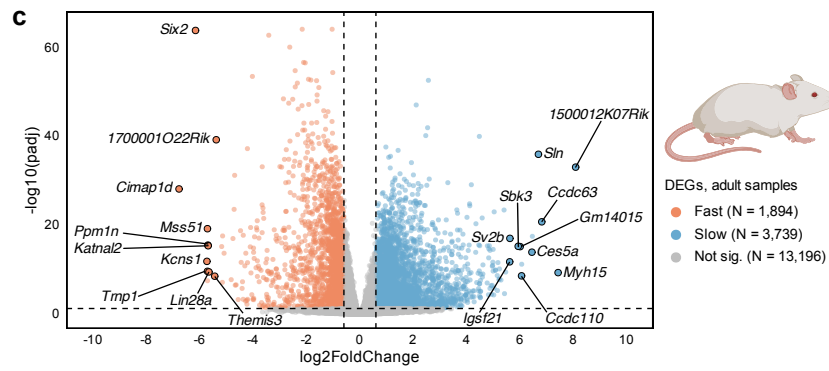
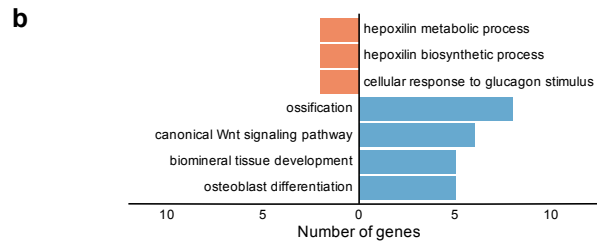
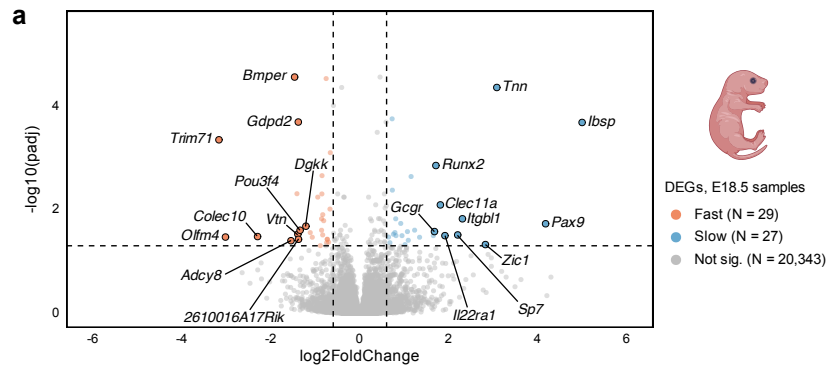
Fig. 4.2 (next page). Differential gene expression between hind limb muscles from distinct anatomical regions at a late embryonic (E18.5) and adult time point. (a) Volcano plot showing DEGs between calf muscles (gastrocnemius and soleus; cyan) and thigh muscles (vastus intermedius and vastus lateralis; gold) at E18.5 ($n = 24$). Gray dots represent genes that did not meet the significance threshold ($|\log_2 \text{fold change}| \geq 0.6$; adjusted $p \leq 0.05$). Gene symbols indicate the top 10 DEGs per anatomical region. (b) Top five enriched GO Biological Process terms for genes significantly up-regulated in calf (cyan) and thigh (gold) muscles at E18.5. (c) Volcano plot showing DEGs between calf and thigh muscles at the adult time point ($n = 23$). (d) Top five enriched GO Biological Process terms for genes significantly up-regulated in calf and thigh muscles at the adult time point. (e) Venn diagram of genes up-regulated in calf muscles, showing those uniquely up-regulated at E18.5 (light cyan), those uniquely up-regulated at the adult time point (dark cyan), or those up-regulated at both time points (genes symbols listed below). (f) Venn diagram of genes up-regulated in thigh muscles, showing those uniquely up-regulated at E18.5 (light gold), those uniquely up-regulated at the adult time point (dark gold), or those up-regulated at both time points (gene symbols listed below).



4.3.3 Genes that distinguish adult myofiber types are not yet differentially expressed between fast- and slow-biased muscles at E18.5.

We next compared DEGs between fast- and slow-biased muscles during myogenesis (E18.5) and in fully differentiated muscles (adult). Since tissue-specific patterns of gene expression during development are important for the formation of adult phenotypes^{493,494}, we hypothesized that DEGs distinguishing fast- and slow-biased muscles at each time point would reflect metabolic pathways and molecular markers of myofiber type. As expected, fewer genes were differentially expressed by myofiber bias at E18.5 ($n = 56$; **Fig. 4.3a**) than in adult muscles ($n = 5,633$; **Fig. 4.3c**). However, DEGs at E18.5 were not enriched for GO terms associated with adult myofiber types but instead reflected broader developmental processes (**Fig. 4.3b**). DEGs in adult muscles reflected the physiological differences between fast and slow myofibers (**Fig. 4.3d**). Specifically, genes up-regulated in adult fast-biased muscles ($n = 1,894$) were enriched for GO terms related to fast myofibers (*Actn3*, *Casq1*, *Mstn*, *Myh4*, *Myod1*, *Tnnc2*, *Tnni2*, *Tnnt3*) and glycolysis (*Gpi1*, *Pgk1*, *Pkm*, *Foxk2*, *Tpi1*, *Gapdh*, *Ldha*). Genes up-regulated in adult slow-biased muscles ($n = 3,739$) were enriched for GO terms related to slow myofibers (*Actn1*, *Myh7b*, *Pdlim1*, *Ppargc1a*, *Tnnc1*, *Tnnt2*) and KEGG pathways related to lipid metabolism (*Acat1*, *Acox1*, *Hadh*) (**Supplementary Excel Table 4.4**). As expected, few DEGs distinguishing fast- and slow-biased muscles were shared across time points. Genes up-regulated in fast-biased muscles across time points ($n = 5$; **Fig. 4.3e**) were associated with lipid metabolism^{495–497}, cell proliferation⁴⁹⁸, and oxidative stress responses⁴⁹⁹. Those up-regulated in slow-biased muscles across time points ($n = 16$; **Fig. 4.3f**) were associated with muscle development^{500–504}, cell proliferation^{505–507}, stem cell renewal^{508,509} and adipose tissue^{510–512}. These results suggest that the gene pathways that distinguish adult myofiber types are not yet fully established at E18.5.

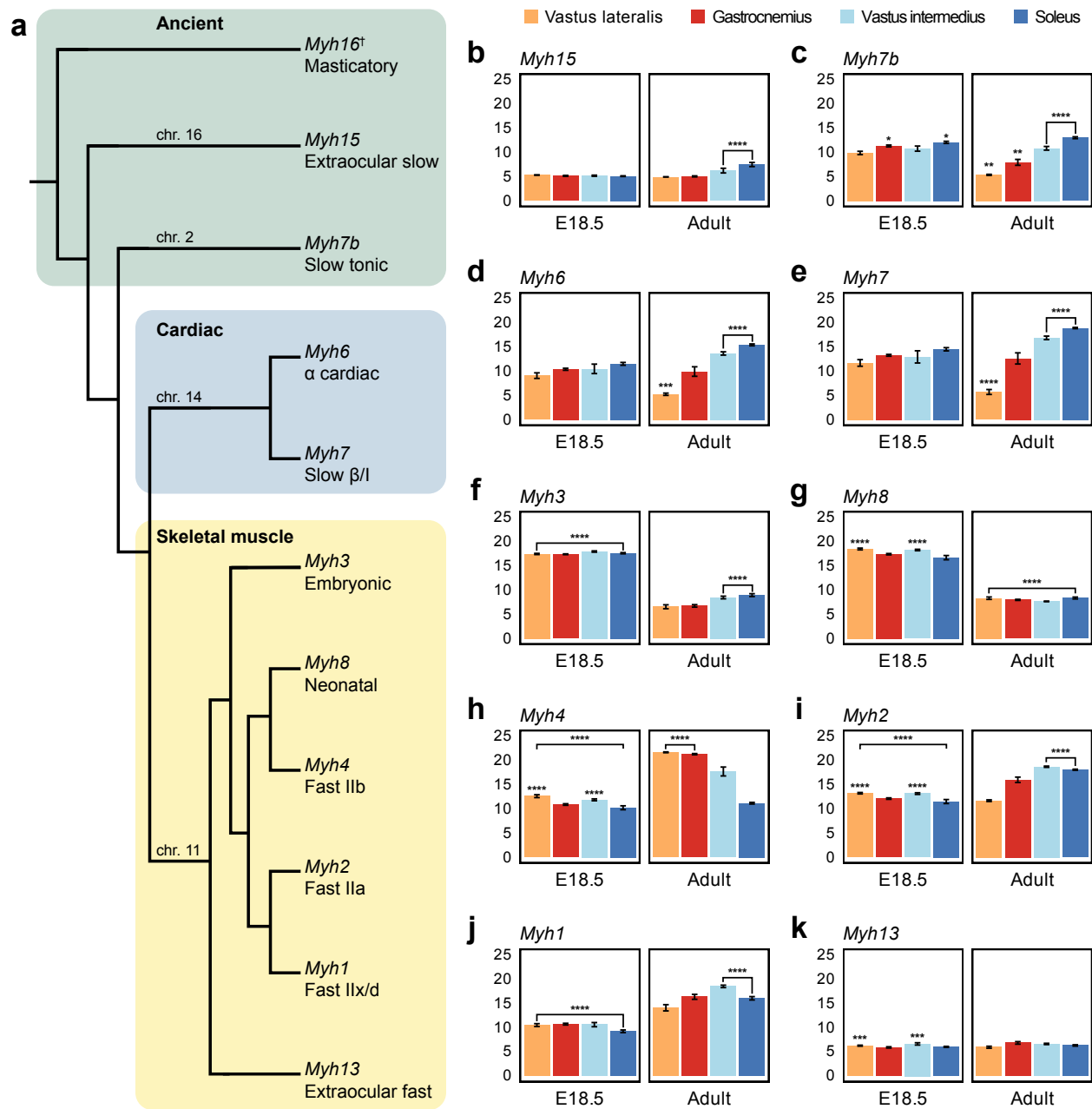
Fig. 4.3 (next page). Analysis of DEGs between fast- and slow-biased muscles at a late embryonic (E18.5) and adult time point. (a) Volcano plot showing DEGs between fast-biased (gastrocnemius and vastus lateralis; orange) and slow-biased (soleus and vastus intermedius; blue) muscles at E18.5 ($n = 24$). Gray dots represent genes that did not meet the significance threshold ($|\log_2 \text{fold change}| \geq 0.6$; adjusted $p \leq 0.05$). Gene symbols represent the top 10 DEGs per myofiber bias. (b) Top five enriched GO Biological Process terms for genes significantly up-regulated in E18.5 fast-biased and slow-biased muscles. (c) Volcano plot showing DEGs between fast- and slow-biased muscles at the adult time point ($n = 23$). (d) Top five enriched GO Biological Process terms for genes significantly up-regulated in adult fast-biased and slow-biased muscles. (e) Venn diagram of genes up-regulated in fast-biased muscles, showing those uniquely up-regulated at E18.5 (light orange), those uniquely up-regulated at the adult time point (dark orange), or those up-regulated at both time points (gene symbols listed below). (f) Venn diagram of genes up-regulated in slow-biased muscles, showing those uniquely up-regulated at E18.5 (light blue), those uniquely up-regulated at the adult time point (dark blue), or those up-regulated at both time points (gene symbols listed below).



4.3.4 Spatiotemporal regulation of MyHC gene expression.

To investigate the molecular composition of myofiber development and differentiation, we quantified the expression of eleven MyHC genes (*Myh16*, *Myh15*, *Myh7b*, *Myh6*, *Myh7*, *Myh13*, *Myh3*, *Myh8*, *Myh4*, *Myh1*, and *Myh2*) in E18.5 and adult muscles (**Appendix H**). Ten MyHC genes were detected, as the ancient masticatory gene *Myh16*^{399,513} was lost in the mouse lineage⁵¹⁴ (**Fig. 4.4a**). As expected, extraocular muscle-associated genes *Myh15* and *Myh13* were the least abundant MyHC gene transcripts in mouse hind limb muscle. Although extraocular slow *Myh15*⁵¹⁵ was up-regulated in adult slow-biased muscles (adjusted $p \leq 0.0001$; **Fig. 4.4b**), the expression of extraocular fast *Myh13* did not significantly vary by time point of myofiber bias (**Fig. 4.4k**). As expected, slow MyHC genes *Myh7b*, *Myh6*, and *Myh7*, which encode the ancient slow tonic, α cardiac, and slow β MyHC proteins, respectively, were up-regulated in adult slow-biased muscles (adjusted $p \leq 0.0001$; **Fig. 4.4c-e**). Notably, *Myh7b* was down-regulated in adult vastus lateralis and gastrocnemius, while *Myh6* and *Myh7* were down-regulated in adult vastus lateralis, alone. Developmental MyHC genes *Myh3* (embryonic) and *Myh8* (neonatal) were up-regulated at E18.5 (adjusted $p \leq 0.0001$) but remained expressed in adult muscle (**Fig. 4.4f,g**). No MyHC genes were significantly differentially expressed between fast- and slow-biased muscles at E18.5, despite the presence of both myofiber types at this stage^{133,437}. Among fast MyHC genes, *Myh4* was up-regulated in adult fast-biased muscles (adjusted $p \leq 0.0001$; **Fig. 4.4h**), whereas *Myh2* (MyHC-IIa) and *Myh1* (MyHC-IIx) were paradoxically up-regulated in adult slow-biased muscles (adjusted $p \leq 0.001$ and ≤ 0.0001 , respectively; **Fig. 4.4i,j**), possibly reflecting differences in promoter accessibility (**Appendix I**). Collectively, these results highlight the time- and muscle-specific regulation of MyHC gene expression.

Fig. 4.4 (next page). Expression dynamics of the striated muscle MyHC gene family. (a) Phylogenetic tree illustrating the relationships among striated muscle MyHC genes and the isoforms they encode. Genes are classified into three groups: ‘Ancient’, ‘Cardiac’ and ‘Skeletal Muscle’. Branch lengths are not to scale. † denotes gene loss in the mouse lineage. (b-k) VST-normalized expression of MyHC genes across mouse hind limb muscles at E18.5 and adult time points. Vastus lateralis and gastrocnemius represent fast-biased muscles; vastus intermedius and soleus represent slow-biased muscles. Asterisks denote statistical significance: * $\text{P}_{\text{adj}} \leq 0.05$; ** $\text{P}_{\text{adj}} \leq 0.01$; *** $\text{P}_{\text{adj}} \leq 0.001$; **** $\text{P}_{\text{adj}} \leq 0.0001$.

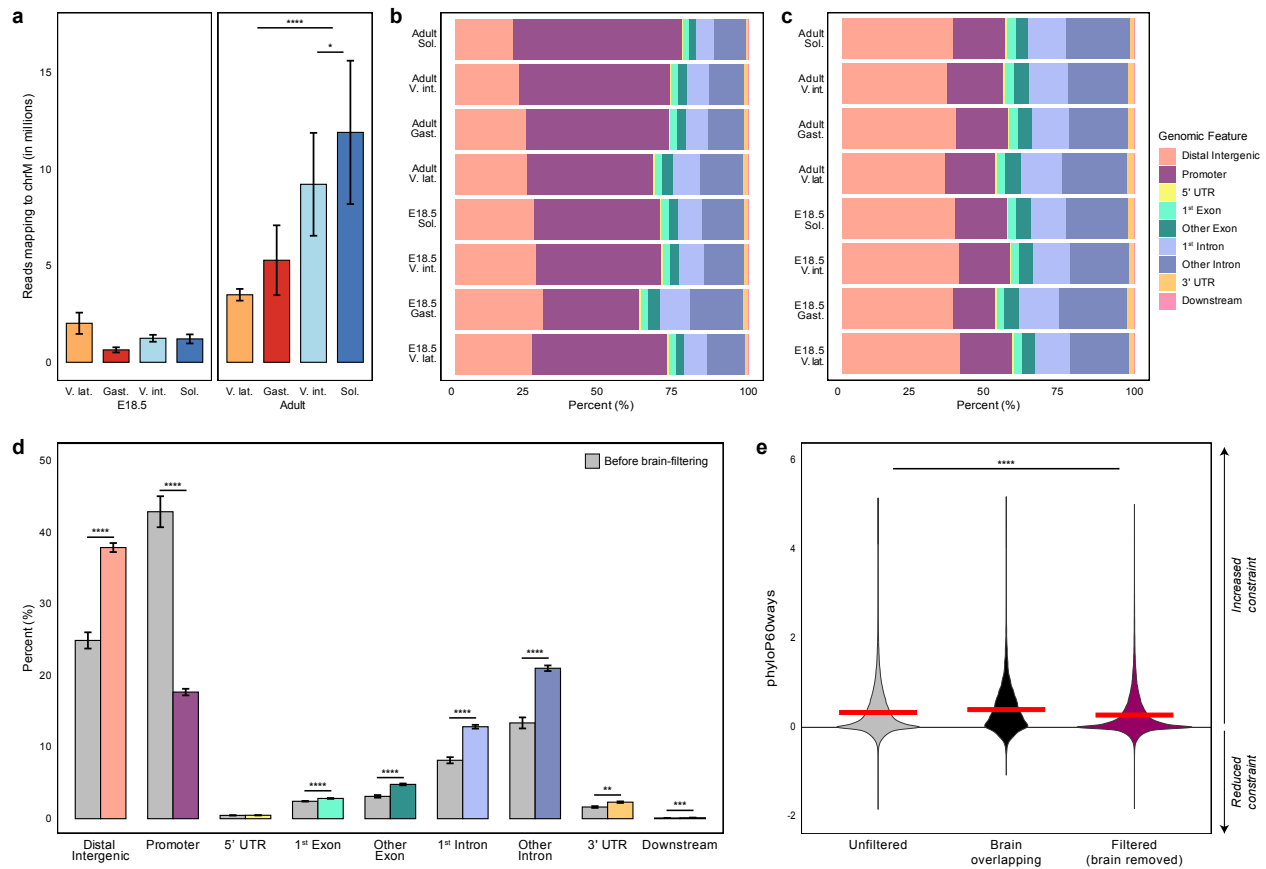


4.3.5 Muscle-specific open chromatin regions are enriched for distal intergenic elements.

We next used ATAC-seq to identify regions of open chromatin (potential CREs) that may regulate DEGs in a myofiber bias- and time point-specific manner⁵¹⁶. Despite using a protocol to reduce mitochondrial content⁴⁵⁵, adult muscles, specifically those with a higher proportion of slow myofibers, were enriched for regions mapping to the mitochondrial genome (chrM) ($\chi^2 = 6.56$, $df = 1$, $p = 0.01041$; **Fig. 4.5a, Supplementary Excel Table 4.2**), likely reflecting increased mitochondrial abundance^{517,518}. No significant difference in chrM mapping was observed at E18.5.

Open chromatin peaks were called using MACS2 and filtered for reproducibility using IDR (**Appendix E**). Most peaks overlapped promoters and distal intergenic regions (i.e., CREs) (**Fig. 4.5b**), with no significant differences in genomic feature composition across IDR peak sets. To enrich for muscle-specific CREs, we excluded peaks showing accessibility in mouse brain⁴⁶³, thereby removing pleiotropic CREs shared across tissue types⁴⁶¹. Brain filtering significantly reduced average peak set size (before: 29,341±10,056; after: 17,122±8,051; $t = 15.907$, $df = 7$, $p = 0.0000009413$), improved GO term enrichment for muscle-related processes (**Appendix J**), and altered genomic feature distributions ($F_{8,126} = 123.8$, $p < 2e-16$; **Fig. 4.5c**, **Appendix K**). After filtering, the proportion of peaks in promoters significantly decreased ($F_{1,14} = 117.9$, $p < 3.34e-08$), while peaks in distal intergenic regions ($F_{1,14} = 100$, $p < 9.33e-08$), first exons ($F_{1,14} = 28.83$, $p < 9.89e-05$), other exons ($F_{1,14} = 56.81$, $p < 2.72e-06$), first introns ($F_{1,14} = 85.69$, $p < 2.41e-07$), other introns ($F_{1,14} = 78.16$, $p < 4.2e-07$), 3' UTRs ($F_{1,14} = 16.79$, $p < 0.0011$), and downstream regions ($F_{1,14} = 22.85$, $p < 0.00029$) significantly increased (**Fig. 4.5d**). Genomic feature distributions were consistent across brain-filtered peak sets. Brain filtering also significantly reduced peak conservation scores (before: 0.3331±0.006; after: 0.2714±0.007; $t = 18.895$, $df = 7$, $p = 2.892e-07$; **Fig. 4.5e**), suggesting that pleiotropic CREs are under greater evolutionary constraint (DNA sequence conservation due to negative selection) than tissue-specific CREs. Overall, this filtering approach enhanced the identification of CREs relevant to muscle biology, a key step toward identifying CREs that shape myofiber development and physiology.

Fig. 4.5 (next page). Chromatin accessibility across E18.5 and adult muscles varying in slow myofiber content ($n = 24$). (a) Average number of ATAC-seq reads aligning to the mitochondrial genome (chrM) by myofiber bias at each time point. Data are presented as mean ± SEM ($n = 3$). (b,c) Proportion of IDR peaks overlapping annotated mm10 genomic features (b) before and (c) after filtering with a mouse brain ATAC-seq peak set. (d) Changes in genomic feature representation following brain filtering. Data are presented as mean ± SEM ($n = 8$). (e) Average per-bp phyloP60ways conservation scores for fixed-length (400 bp) IDR peaks. Red lines indicate the mean conservation score for each peak set. Positive values reflect increased evolutionary constraint (slower-than-expected DNA sequence change across mammals), while negative values indicate reduced evolutionary constraint (faster-than-expected DNA sequence change across mammals). Asterisks denote statistical significance: * $P \leq 0.05$; ** $P \leq 0.01$. *** $P \leq 0.001$; **** $P \leq 0.0001$. Abbreviations: V. lat., vastus lateralis; Gast., gastrocnemius; V. int., vastus intermedius; Sol., soleus.



4.3.6 Condition-specific peak sets are enriched with TF motifs important for myogenesis and myofiber differentiation.

To identify candidate CREs that may regulate genes influencing myofiber type, we distilled 136,976 brain-filtered peaks into nine non-overlapping ‘condition-specific’ peak sets. These included peaks unique to muscles of a specific time point and myofiber bias (E18.5 Slow, Adult Slow, E18.5 Fast and Adult Fast), peaks shared across all muscles of a given time point (E18.5 and Adult) or myofiber bias (Slow and Fast), and peaks shared across all muscles (Universal) (**Fig. 4.6a**). Only 25,436 peaks (18.6%) fell into these categories (**Supplementary Excel Table 4.5**), with most exclusive to E18.5 muscles ($n = 15,294$). More peaks were shared across Adult Fast-biased muscles ($n = 3,641$) than Adult Slow-biased muscles ($n = 1,470$). Many peaks were Universal ($n = 3,005$), which likely represent pleiotropic CREs regulating core muscle functions. Indeed, genes near Universal peaks were enriched for GO terms related to muscle physiology, metabolism, and development (**Supplementary Excel Table 4.6, Appendix L**). Few peaks were shared across time points in Slow-biased ($n = 78$) or Fast-biased ($n = 231$) muscles, suggesting limited overlap in gene regulation across time points. Genomic feature distributions were consistent across condition-specific peak sets and similar to brain-filtered peak sets (**Appendix M**).

We next assessed sequence conservation across condition-specific peak sets, hypothesizing that peaks shared by all muscles from the same time point (E18.5 and Adult) and peaks shared across time points (Universal, Fast, and Slow) would be more conserved than peaks unique to a specific time point and myofiber bias. Average per-bp conservation scores were generally positive (0.220 ± 0.003), indicating some degree of evolutionary constraint across condition-specific peak sets (**Fig. 4.6b**). E18.5 (0.290 ± 0.006), Adult (0.272 ± 0.01), Universal (0.268 ± 0.008), E18.5 Fast (0.193 ± 0.007), Adult Slow (0.164 ± 0.01) and Adult Fast (0.162 ± 0.007) sets were significantly more conserved than a random background ($p = 0.03126$), with E18.5, Adult, and Universal sets being the most conserved (**Fig. 4.6c**). E18.5 Slow (0.134 ± 0.006), Fast (0.093 ± 0.02) and Slow (0.08 ± 0.04) sets did not significantly differ from the random background.

Finally, we examined transcription factor (TF) motif enrichment, hypothesizing that condition-specific peak sets would be enriched for TFs involved in myogenesis and myofiber differentiation. E18.5 peak sets were enriched for bHLH and bZIP motifs (**Fig. 4.6d**), which regulate muscle development and differentiation^{519,520}. Adult peak sets were enriched for MADS and NR motifs, which control muscle growth, regeneration, and metabolism^{521–524}. The Universal peak set, which likely represents pleiotropic CREs active across all muscles across time points, was enriched for both bZIP and MADS motifs, consistent with a role in maintaining muscle cell identity and function throughout life^{525–527}.

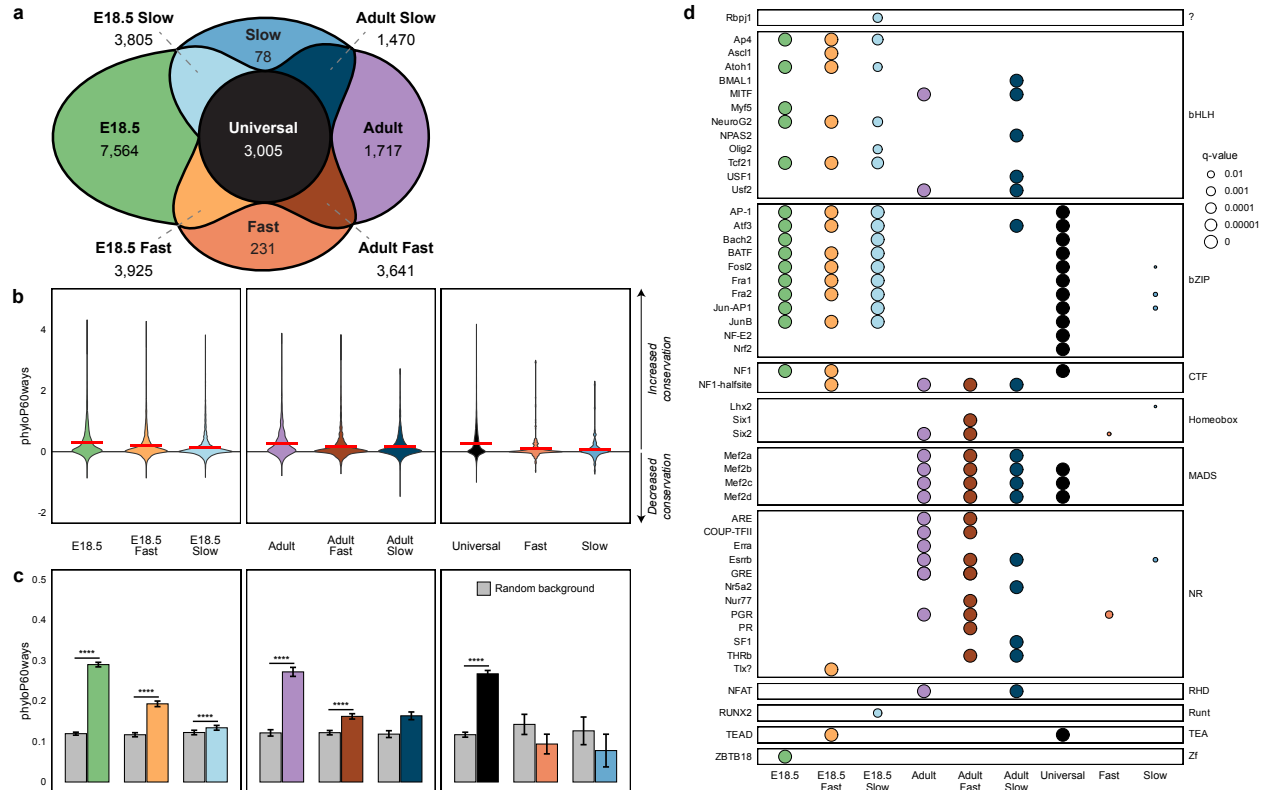


Fig. 4.6. Characterization of condition-specific peak sets. (a) Venn diagram showing the number of open chromatin regions in each of the nine condition-specific peak sets. (b) Average per-bp phyloP60ways conservation scores for fixed-length (400 bp) condition-specific peaks. Red lines indicate the mean conservation score for each peak set. Positive values reflect increased evolutionary constraint (slower-than-expected DNA sequence change across mammals), while negative values indicate reduced evolutionary constraint (faster-than-expected DNA sequence change across mammals). (c) Average per-bp phyloP60ways conservation scores for each peak set compared to a random background (grey bars). Asterisks denote significant differences from a random background: **** $p \leq 0.0001$. (d) Top 15 enriched transcription factor (TF) binding site motifs identified in each peak set, grouped by TF family.

4.3.7 Nine candidate CREs enhance and three suppress luciferase activity in muscle cells.

To target CREs that may underlie the distinct properties of adult myofiber types, we used condition-specific peak sets to annotate open chromatin regions surrounding DEGs between adult fast- and slow-biased muscles (see example in **Appendix F**). We prioritized peaks with accessibility patterns mirroring the expression of nearby (<100 kb) DEGs (e.g., peaks accessible only in adult slow-biased muscles near genes up-regulated only in adult slow-biased muscles) and exhibiting elevated per-bp conservation scores^{528,529}. This approach identified 12 candidate CREs: nine associated with fast-biased muscles and three with slow-biased muscles (**Supplementary Excel Table 4.3**). Each candidate was functionally validated using a dual luciferase reporter assay in a mouse muscle cell line. Results were consistent across experiments (Fisher's combined probability test, $p \leq 0.000000004$). Nine candidates significantly

increased luciferase activity relative to the empty control vector (**Fig. 4.7**), with four exceeding a 2-fold increase ($p \leq 0.0001$): Slow 2 ($\log_2\text{FC} = 4.28$), Fast 1 (3.58), Slow 3 (2.38) and Fast 3 (1.66). In contrast, candidates Fast 8, Fast 9, and Slow 1 significantly suppressed luciferase activity (negative $\log_2\text{FC}$, $p \leq 0.0001$). These results suggest that candidates Fast 1-7, Slow 2, and Slow 3 may function as enhancers, while candidates Fast 8, Fast 9, and Slow 1 may function as silencers. However, the direct effect of each candidate on its putative target gene remains unknown.

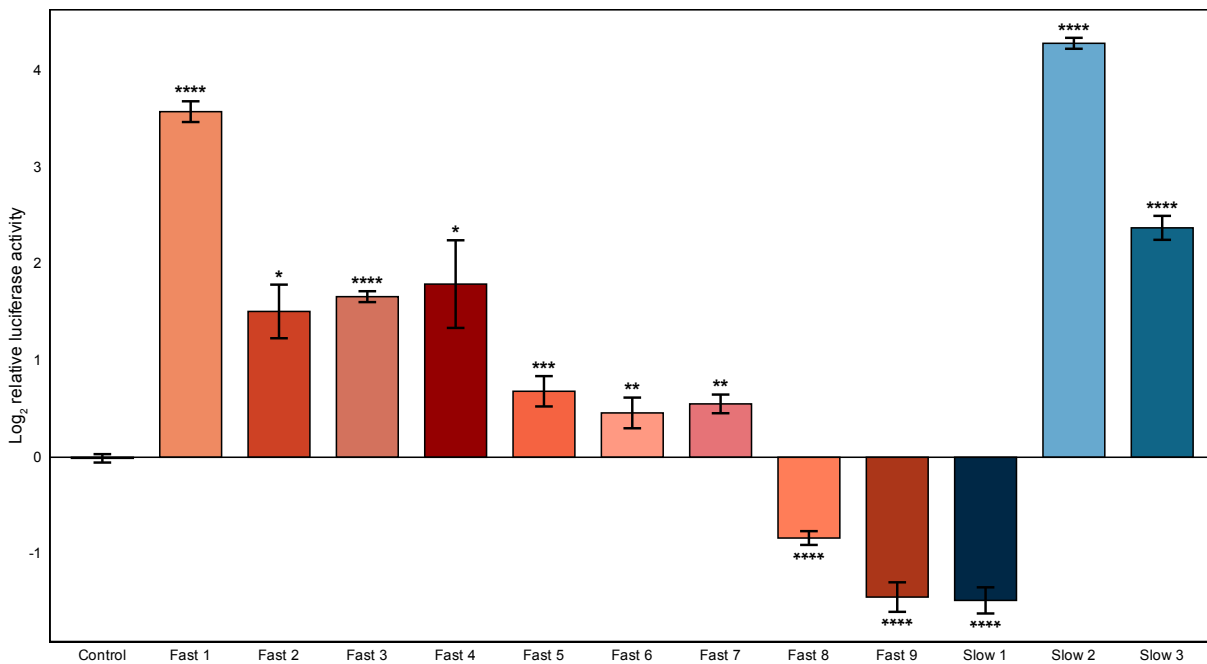


Fig. 4.7. Functional validation of candidate CREs using a dual-luciferase reporter assay in C2C12 cells. Relative luciferase activity driven by 12 candidate CREs in mouse muscle cells. CREs associated with fast myofiber gene pathways are shown in orange; those associated with slow myofiber gene pathways are shown in blue. Positive $\log_2$ fold change ($\log_2\text{FC}$) values indicate enhanced luciferase activity, while negative values indicate suppressed luciferase activity relative to the empty pGL4.23 control vector. Data are presented as means $\pm$ SEM ($n = 8$). Asterisks denote statistically significant differences from the control vector: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

4.4 Discussion

Here, we used RNA-seq and ATAC-seq to characterize the regulatory landscape of mouse hind limb muscles that differ in slow myofiber content at a late embryonic and adult time point. By integrating patterns of gene expression and chromatin accessibility, we uncovered key biological processes driving dynamic changes in gene regulation across tissues and time points, providing new insights into

myogenesis and the molecular establishment of muscle identity. Our results suggest that distal CREs play a central role in orchestrating tissue and time-specific patterns of gene expression during myogenesis and myofiber differentiation. Through this integrative approach, we identified twelve candidate CREs (nine enhancers and three silencers) that may regulate fast and slow myofiber gene pathways in a time point- and muscle-specific manner, ultimately shaping potential adult muscle function.

Given the distinct biological processes occurring in developing versus fully developed muscle, most variation in gene expression and chromatin accessibility was attributed to time point. Embryonic muscles exhibited significantly more DEGs and open chromatin regions, consistent with previous studies highlighting the dynamic regulatory landscape of developing tissue^{530,531}. As expected, few genes up-regulated at E18.5 remained so in adulthood, emphasizing the time point-specific nature of gene regulation during development^{532–537}. Exceptions, such as *Hoxc6* and *Hoxc8* in thigh musculature, highlight the importance of such genes in maintaining muscle identity throughout life^{538,539}. One of the defining features of embryonic development is limb patterning⁵⁴⁰, which relies on the precise spatiotemporal expression of genes that define limb segments and establish muscle identity⁴⁷⁷. Accordingly, gene expression patterns in embryonic muscles primarily reflected their anatomical location within the hind limb (e.g., calf vs. thigh). Chromatin accessibility patterns, however, did not show this anatomical specificity, suggesting that limb muscle development may be regulated by a shared set of pleiotropic CREs or super-enhancers^{541–543}.

In adult muscles, patterns of gene expression and chromatin accessibility primarily reflected differences in myofiber metabolism and contractile properties. DEGs and accessible chromatin regions were enriched for pathways characteristic of each myofiber type: glycolytic pathways in fast-biased muscles, and mitochondrial biogenesis and lipid metabolism in slow-biased muscles. These metabolic differences were not observed in E18.5 muscles, suggesting that such pathways are not yet differentially regulated between myofiber types at this stage. Adult slow-biased muscles exhibited greater mitochondrial read abundance, consistent with the high mitochondrial content of slow myofibers, which rely on oxidative phosphorylation for energy^{139–141,544}. In contrast, E18.5 muscles displayed low mitochondrial read abundance, indicating that developing myofibers lack the mitochondrial density and metabolic activity typical of mature myofibers^{139–141}. It is expected that as differentiation and muscle activation patterns progress, mitochondrial content and the expression of genes involved in lipid metabolism will dramatically increase⁵⁴⁵, consistent with the enrichment of these pathways in adult muscles.

As expected, expression of the slow MyHC genes *Myh6*, *Myh7* and *Myh7b* was highest in adult slow-biased muscles (soleus, 58% slow; vastus intermedius, 45% slow), whereas expression of fast *Myh4* peaked in adult fast-biased muscles (gastrocnemius, 8% slow; vastus intermedius, 0% slow)¹⁵⁶. *Myh4* was

the most highly expressed MyHC gene, unsurprising given that the muscles of small-bodied mammals like mice are enriched with MyHC-IIb^{159–162,546}. Although transcripts of both fast and slow MyHC genes were detected in embryonic muscle, they were not yet differentially expressed by myofiber bias, suggesting that these pathways are only beginning to be activated. This is consistent with previous studies showing that fast and slow myofibers do not significantly differentiate until after birth, coinciding with increased muscle innervation^{223,224,430,431,547}. Interestingly, expression of *Myh2* and *Myh4* was significantly elevated in E18.5 thigh muscles compared to E18.5 calf muscles, suggesting that myofiber differentiation may occur earlier in the thigh^{478,479}. Unexpectedly, *Myh2* and *Myh1* showed the highest expression in adult slow-biased muscles. Although these isoforms are known to be highly expressed in the mouse soleus⁵⁴⁸ and may be associated with slow myofiber composition⁵⁴⁹, this expression pattern likely reflects the substantial proportion of fast myofibers typically observed in slow-biased mouse muscles^{156,432}. Of note was the elevated expression of *Myh15* in adult slow-biased muscles. Although transcripts have been detected beyond extraocular muscles^{399,550–552} this pattern may reflect coordinated sensory-motor function in intact muscle with varying contractile phenotype.

Of particular interest to understanding the observed variation in fast MyHC gene expression is the open chromatin landscape surrounding the fast MyHC gene cluster. While the fast MyHC super-enhancer^{195,196} was accessible in both embryonic and adult muscle, individual fast MyHC gene promoter accessibility varied. The *Myh4* promoter was broadly accessible across adult muscles with larger populations of fast myofibers (i.e., gastrocnemius, vastus lateralis and vastus intermedius), with a secondary promoter uniquely accessible in the adult gastrocnemius, correlating with elevated *Myh4* expression in this muscle. In contrast, the *Myh2* promoter was accessible only in the adult vastus intermedius, which also exhibited the highest *Myh2* expression. *Myh1* promoter accessibility was more widespread, particularly in the adult soleus and vastus intermedius, potentially explaining the elevated expression of *Myh1* in these slow-biased muscles. In embryonic muscles, both promoter accessibility and gene expression of *Myh2*, *Myh1*, and *Myh4* were minimal, indicating that super-enhancer activity alone is insufficient to drive strong gene expression. In comparison, the promoter of prenatal *Myh8* was accessible across embryonic muscles, consistent with its elevated expression at E18.5. These findings suggest that accessibility of the fast MyHC super-enhancer alone does not reliably predict gene expression. Instead, expression depends on both promoter accessibility and the binding of regulatory proteins that mediate enhancer-promoter interactions⁵⁵³. Consequently, species-specific differences in muscle myofiber composition may arise from coordinated changes not only to CRE sequences that affect regulatory protein binding, but to the expression of genes that regulate the accessibility of CREs throughout development via epigenetic modifications^{554,555}.

Uncovering the genetic basis of muscle myofiber composition requires identifying CREs active

during muscle development that govern the differentiation and function of fast and slow myofibers. By targeting regions of open chromatin with high specificity to muscle tissue, we identified candidate CREs (condition-specific peak sets) that may drive the expression of DEGs in a time point- and muscle-specific manner. Because fast and slow myofiber pathways do not significantly diverge until after birth, key CREs are likely found within peak sets unique to adult fast- and slow-biased muscles. Interestingly, the ‘Adult Slow’, ‘Adult Fast’, ‘Fast’, and ‘Slow’ condition-specific peaks exhibited the lowest conservation scores, suggesting that these regions are under relaxed evolutionary constraint and more prone to DNA sequence change, which may contribute to myofiber composition variation both within and between species. Such regions are especially relevant to studies of locomotor evolution, as mutations in CREs that alter TF binding, and thus the timing and location of gene expression, are a well-established source of evolutionary novelty and phenotypic diversity¹⁸⁵. CREs unique to specific cell types or tissues are predicted to evolve more rapidly than those shared across cell types, highlighting their potential to shape species-specific traits⁵⁵⁶. In support of this, we identified twelve muscle-specific CREs (nine enhancers and three silencers) associated with fast and slow myofiber pathways that may influence myofiber differentiation and phenotype. Species-specific variation within these CREs may underlie differences in myofiber composition and contribute to the evolution of diverse locomotor strategies that benefit from muscles with distinct proportions of fast and slow myofibers (e.g., humans with slow-biased lower limb muscles; moles with fast-biased shoulder musculature)^{423,557}. It is tempting to speculate that fast myofiber pathways may be promoted through the activity of Fast 1-7 and Slow 1, whereas slow myofiber pathways may be promoted through the activity of Slow 2-3 and Fast 8-9. Furthermore, human-specific mutations that amplify slow myofiber pathways by enhancing slow CRE activity or repressing fast CRE activity, may have been under positive selection in the human lineage.

Our results offer a snapshot of the regulatory landscape underlying muscle development at a late embryonic and adult time point, highlighting the spatiotemporal regulation of limb muscle patterning and the refinement of adult muscle identity. We identified twelve muscle-specific CREs, nine enhancers and three silencers, that drive gene expression *in vitro* and may contribute to the phenotypic differences observed between fast and slow myofibers. These CREs represent compelling targets for further analysis, particularly in relation to myofiber composition and locomotor performance. Testing the regulatory function of these CREs during myogenesis and evaluating the impact of species-specific sequence variation on target gene expression will help elucidate the genetic basis of species-specific differences in muscle myofiber content.

In this study, MyHC isoform levels were not directly measured, so we relied on the consistent and widely reported patterns of MyHC isoform expression in murine muscle for our interpretation⁵⁵⁷. Rodent muscle, which tends to exhibit relatively homogenous MyHC isoform expression across myofibers

compared to humans, provided a useful system for investigating how CREs regulate myogenesis and myofiber differentiation. Because RNA-seq and ATAC-seq datasets were not matched, direct comparisons between these two data sets are limited. Epigenetic profiles were also not generated from single cells^{558,559}, so signals specific to fast and slow myofibers may be obscured by the diverse cell types present in muscle tissue^{560,561}. Therefore, future studies using matched scRNA-seq and scATAC-seq samples on single myofibers with known MyHC content will be essential to extending these findings.

4.5 Bridge

Given the genetic basis of muscle myofiber composition, Chapter 4 used a mouse model to investigate CREs that regulate myofiber type as a first step toward understanding human muscle evolution. We characterized the regulatory landscape of late developmental (E18.5) and adult hind limb muscles from two anatomical compartments (calf and thigh) that differ in slow myofiber content, identifying thousands of muscle-specific CREs unique to fast- and slow-biased muscles, 12 of which were shown to affect gene expression *in vitro*. Building on this, Chapter 5 hypothesizes that some of these CREs are also functional in humans (i.e., evolutionarily conserved mammalian CREs) and contain human-specific variants that promote slow myofiber pathways, contributing to slow-biased muscles.

5 IDENTIFYING POTENTIAL *CIS*-REGULATORY ELEMENTS THAT MAY SHAPE HUMAN MUSCLE EVOLUTION

5.1 Introduction

Among primates, humans are unique in being habitual bipeds (i.e., exclusively walking on two limbs) and are the only extant species capable of endurance running¹. Quadrupedal chimpanzees (*Pan troglodytes*) are unable to match the locomotor economy of human walking^{2,3}, and over marathon-length distances humans can outrun almost all animals, including horses, especially in hot conditions⁴. Human locomotor endurance has relied on 7-8 million years of fine-tuning adaptations to the primate spine, pelvis, and lower limb, which are easily traced in the fossil record^{1,71,72}. However, many traits essential for long-distance bipedal locomotion are not preserved in the fossil record, making their evolutionary origins difficult to reconstruct^{1,59,105–109}. Of particular interest is the unusually high proportion of fatigue-resistant slow myofibers in human lower limb muscles, which enhance endurance and underlie species-specific differences in muscle performance^{2,167,424}. Selection for more efficient bipedal locomotion likely shaped hominin muscle physiology over the past 7-8 million years, resulting in the slow-biased lower limb musculature observed in modern humans^{13,108,131}. Although muscles themselves are not typically preserved in the fossil record, the genome provides a valuable tool for investigating the genetic basis of fatigue-resistant muscles and identifying potential targets of selection.

Given the high similarity of protein-coding (gene) sequences between humans and chimpanzees, phenotypic differences are largely attributed to species-specific variation in evolutionarily conserved non-coding regions of the genome¹⁷². These regions often act as spatiotemporal regulators of gene expression (e.g., *cis*-regulatory elements, CREs, such as enhancers that enhance gene activity and silencers that silence gene activity) that control where, when, and how much a gene is expressed during development¹⁷³. Mutations in CREs can alter the timing or location of gene expression at critical developmental stages, producing phenotypic variation and driving evolutionary divergence between species^{174–184}, including the emergence of human-specific traits^{187–194}. Thus, human-specific mutations in evolutionarily conserved CREs (i.e., CREs preserved from the common ancestor of humans and other mammals) that regulate genes influencing myofiber development and muscle physiology may be key to understanding the emergence of slow-biased muscles and the evolution of endurance locomotion.

Here we used a mouse model to identify evolutionarily conserved mammalian CREs under accelerated evolution in the human lineage that may have contributed to the evolution of hominin endurance locomotion. Using evolutionary developmental genomic approaches, we identified 190 candidate CREs active in human striated muscle and overlapping regions of human-specific accelerated

evolution. Six candidates were chosen for functional validation by testing the effects of human- and chimpanzee-specific sequence variation on gene expression in a human skeletal muscle cell line. While our approach identified nine active enhancers, none displayed consistent differences between human and chimpanzee sequences. These results may highlight the need for more nuanced experiments that capture the native regulatory environment of primate muscle, as well as functional validation of the remaining CRE candidates ($n = 184$), to fully evaluate the role of skeletal muscle endurance in the evolution of hominin locomotion.

5.2 Methods

5.2.1 Identification of skeletal muscle-specific cis-regulatory elements (CREs) using a mouse model

This chapter uses RNA-seq and brain-filtered ATAC-seq datasets generated from mouse muscle samples, as described in Chapter 4, to identify CREs that may regulate the development and expression of skeletal muscle phenotypes (**Fig. 5.1**). Detailed descriptions of sample preparation, sequencing and data processing for both datasets can be found in Chapter 4.2.2 and 4.2.4 (Methods). Briefly, ATAC-seq was used to profile peaks of open chromatin accessible to transcriptional machinery, representing potential CREs^{198,199}. To reduce background noise and exclude peaks likely corresponding to pleiotropic CREs active across multiple tissues, a series of filtering steps were applied (Chapter 4.2.5), resulting in the brain-filtered ATAC-seq dataset used here ($n = 44,644$ peaks). Although some open chromatin peaks shared between brain and muscle may contribute to muscle biology, this conservative filtering strategy was used to bias our dataset toward CREs with greater muscle specificity. In addition, RNA-seq was used to identify genes differentially expressed across time points, myofiber biases, anatomical regions, and muscle identities (Chapter 4.2.3). These datasets were then integrated to target differentially accessible CRE candidates potentially driving observed differences in gene expression.

5.2.2 Mapping condition-specific peaks to the human *hg19* genome

To identify mouse CREs that were also functional in humans (i.e., evolutionarily conserved mammalian CREs), mouse (mm10) peak coordinates were first mapped to orthologous human (hg19) coordinates. To increase the proportion of peaks identified in Chapter 4 ($n = 44,644$) that map to the human genome, condition-specific peak sets (i.e., Adult, E18.5, Universal, E18.5 Fast, E18.5 Slow, Adult Fast, Adult Slow, Fast, Slow, E18.5 Adult Fast, E18.5 Adult Slow, E18.5 Adult Soleus, E18.5 Adult Not Soleus, E18.5 Calf, E18.5 Thigh, Adult Calf, Adult Thigh, Calf, and Thigh) were divided into 200 bp windows and converted to orthologous human (hg19) coordinates using the LiftOver tool from the University of California Santa Cruz (UCSC) Genome Browser⁵⁶². The proportion of reads mapping to

hg19 was then compared against that of a matched random background constructed to exclude mm10 repeat elements and blacklist regions using binomial logistic regression.

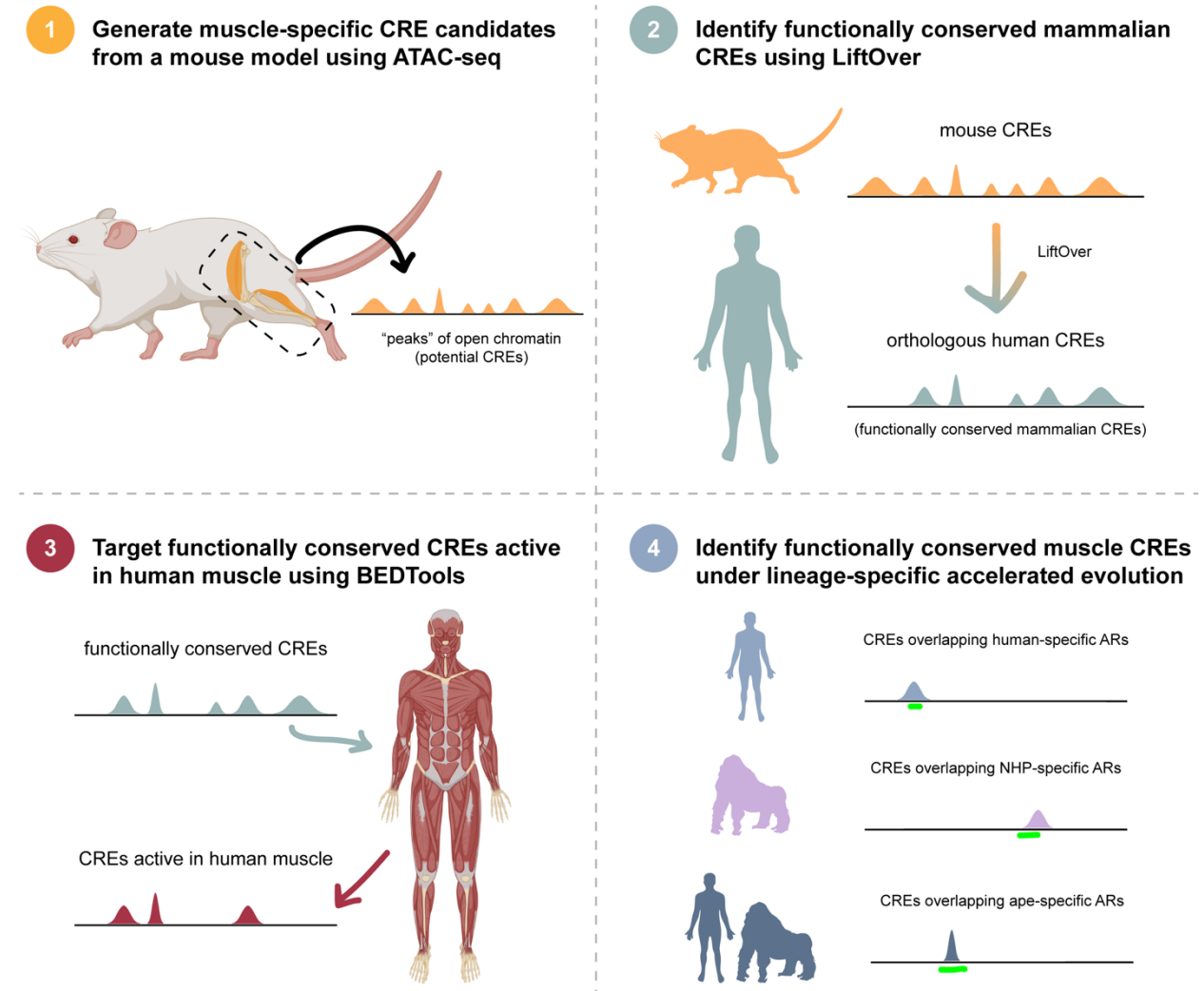


Fig. 5.1. Schematic illustrating the workflow for identifying muscle-specific CREs that are evolutionarily conserved across mammals and exhibit signatures of accelerated evolution, using ATAC-seq datasets generated from mice. 'AR' denotes accelerated regions.

To assess inter-species conservation, phyloP⁵⁶³ scores for 60 mammalian species (mm10.60way.phyloP60way.bw) and 100 vertebrate species (hg19.100way.phyloP100way.bw) were obtained from the UCSC Genome Browser (<http://genome.ucsc.edu>)⁴⁶⁶ and used to extract per-base conservation scores for peaks that either failed or successfully mapped to the human (hg19) genome. To control for variation in peak length across peak sets, all peaks were resized to a fixed width of 400 bp, centered ± 200 bp around the peak midpoint. Per-base conservation scores were averaged across each

fixed-width peak, and these mean peak conservation scores were then averaged across each condition-specific peak set to generate an overall peak set conservation score. Conservation scores from mapped and unmapped peaks were compared using a paired *t*-test and a Wilcoxon rank-sum test. Peaks were further annotated to genomic features using the R package ChIPseeker (v.1.36.0)⁴⁶⁷, defining promoters as $\pm 1,000$ bp around the transcription start site (tssRegion = c(-1000, 1000)). Significant differences in genomic feature representation were assessed using a G-test and an ANOVA with Tukey's post hoc test. Visualizations were created using the R package ggplot2 (v.3.5.1)⁴⁵⁰.

5.2.3 Intersections with human DNA-seq datasets

To identify evolutionarily conserved mammalian CREs active in human muscle, we intersected human-converted peaks with CRE signals generated from human tissue using DNase-seq. DNase-seq, like ATAC-seq, profiles open chromatin regions accessible to transcriptional machinery (i.e., DNase I hypersensitivity sites, DHSs) that may function as CREs^{564,565}. DNA-seq data from 17 human tissue types from both prenatal (post-day 100) and postnatal time points were obtained from the NIH Roadmap Epigenomics Consortium⁵⁶⁶. See **Supplemental Table S1** for sample details and accession numbers. BED files were processed with MACS3 (v.3.0.3)⁴⁵⁸ to call peaks, using the parameters -B -f BED -g hs -q 0.01 --shift -75 --extsize 150 --nomodel --keep-dup all. Peak output files were then merged into non-redundant consensus sets for each tissue and time point (e.g., Adult Heart, Fetal Heart) using the intersect function from BEDTools.

Merged MACS3 peak output files were intersected using the multiinter function from BEDTools to generate 20 non-overlapping, tissue- and time point-specific open chromatin peak sets (i.e., Fetal Brain, Fetal Spinal Cord, Fetal Heart, Adult Heart, Fetal Lung, Fetal Thymus, Fetal Pancreas, Fetal Adrenal Gland, Fetal Kidney, Fetal Stomach, Adult Gastric Mucosa, Fetal Small Intestine, Adult Small Intestine, Fetal Large Intestine, Fetal Ovary, Adult Ovary, Fetal Testes, Fetal Arm Muscle, Fetal Leg Muscle, and Adult Psoas Muscle). Condition-specific peak sets were then intersected with these human tissue peak sets using the intersect function from BEDTools to calculate the proportion of condition-specific peaks accessible in each tissue and time point. Significant differences in tissue overlap were determined using the Kruskal Wallis test, Wilcoxon rank-sum test, and beta regression.

A non-redundant 'striated muscle' peak set, representing regions accessible in each of the cardiac (heart) and skeletal muscle (leg, arm, and psoas) MACS3 peak output files, was generated using the merge function from BEDTools. Condition-specific peak sets were then intersected with this striated muscle peak set using BEDTools intersect to define 'striated muscle-accessible' peaks. Differences in overlap with the striated muscle peak set were compared using the Kruskal Wallis Test and Wilcoxon rank-sum test. Differences in peak conservation scores and ChIPseeker annotations were evaluated as

described above. Peaks were further annotated to nearby genes and analyzed for functional enrichment using the Genomic Regions Enrichment of Annotations Tool (GREAT, v.4.0.4)⁴⁶⁸.

5.2.4 Accelerated region analysis

To identify candidates with potential relevance to primate evolution, we compiled 13 previously published evolutionary datasets for intersection with CREs active in human muscle (**Supplemental Table S2**)^{405,411,567–577}. These datasets contain thousands of genomic regions showing evidence of accelerated evolution in humans, extant non-human primates, and stem primate lineages (e.g., primate, catarrhine, ape), which are informative for human evolution. Such regions are thought to harbor CREs that drive lineage-specific changes in gene expression that contribute to differences in phenotype⁵⁷⁸. The datasets encompass both species-specific and lineage-specific accelerated regions (ARs), which are evolutionarily conserved DNA sequences exhibiting an excess of DNA substitutions in a given lineage. These include HARs (human accelerated regions), CHARs (chimpanzee accelerated regions), other lineage-specific ARs (linARs; e.g., gibbon, howler monkey, anthropoid, hominini), and PARs (primate accelerated regions, spanning humans and non-human primates), as well as additional classes such as HAQERs (human ancestor quickly evolved regions), haDHSs (human accelerated DNase I hypersensitivity sites), ANCs (accelerated conserved non-coding sequences), HACNs (human-accelerated non-coding sequences), and HTBEs (human terminal branch elements). We also included human-specific structural modifications within conserved non-coding regions, including hCONDELs (conserved human-specific deletions), fhINSs (fixed human-specific insertions), and fhDELs (fixed human-specific deletions). These represent human-specific sequence gains or losses of varying lengths within otherwise conserved regions that are believed to affect gene expression^{405,567,579}. All genomic coordinates were converted to the human hg19 genome using LiftOver.

Striated muscle-accessible peak sets were intersected with each hg19 evolutionary dataset using BEDTools intersect. Evolutionary dataset enrichment was compared to a random background ($n = 1,000$ permutations) using a paired t -test. All peak sets were annotated to genomic features and nearby genes using ChIPseeker, with differences in feature representation assessed via G-test and ANOVA with Tukey's post hoc test. Transcription factor (TF) motif enrichment was evaluated using Hypergeometric Optimization of Motif EnRichment (HOMER, v. 4.10.4)⁴⁶⁹, with the parameters findMotifsGenome.pl -size given -mask.

To identify CREs potentially regulating muscle-associated genes, we extracted genes located within a $\pm 500,000$ bp *cis*-regulatory window^{580–583} surrounding each accelerated region-overlapping peak using the R packages GenomicRanges (v.1.60.0)⁵⁸⁴, biomaRt (v.2.64.0)⁵⁸⁵, and AnnotationDbi (v.1.70.0). Genes within these windows associated with muscle-related gene ontology (GO) terms were identified using

GO.db (v.3.21.0) and the case-insensitive keywords "muscle", "myo", "actin", "sarcomere", "mitochondria", "oxid", "fiber", "filament", "fibril", "contractile", "lipid", and "metaboli". GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed on all genes identified within *cis*-regulatory windows using the R package clusterProfiler (v.4.16.0)⁴⁵¹, and redundant GO terms were reduced using the clusterProfiler function simplify(). Human disease ontology (DO) analysis was performed using the R package EnrichDO (v.1.2.0)⁵⁸⁶. All statistical analyses used an adjusted p-value ≤ 0.05 . Evolutionary dataset-overlapping peaks were then intersected with publicly available hg19 single nucleotide polymorphism (SNP) datasets (ClinVar⁵⁸⁷ and Common dbSNP release 155⁵⁸⁸) using BEDTools, and SNP enrichment was compared to a random background ($n = 1,000$ permutations) using a paired *t*-test.

5.2.5 Candidate CRE selection for functional analysis

To visually assess the epigenetic landscape surrounding striated muscle-accessible peak sets overlapping evolutionary datasets, peak set BED files were imported into the UCSC Genome Browser. The following annotation tracks were loaded: Base Position, GENCODE VM23, NCBI RefSeq, ENCODE cCREs, ORegAnno, ENCODE regulation, CpG Islands, JASPAR Transcription Factors, FANTOM5, RefSeq Func Elems, ReMap ChIP-seq, Placental Mammal Basewise Conservation by PhyloP (PlacentalCons), and RepeatMasker. Candidate CREs were prioritized based on multiple criteria, including overlap with human-specific regions of accelerated evolution (e.g., HARS, hCONDELs), chromatin accessibility patterns across ATAC-seq datasets, nearby gene function, nearby gene expression patterns derived from RNA-seq, strength of regulatory signal from UCSC Genome Browser tracks (e.g., ENCODE CREs), mammalian conservation scores (phyloP60ways), and presence of human-specific sequence change. High priority was given to candidates exhibiting strong regulatory signals and high evolutionary conservation located within ± 100 kb of DEGs known to influence fast and/or slow myofiber phenotypes and muscle physiology. Of particular interest were candidates accessible exclusively in slow-biased peak sets and located near slow myofiber genes up-regulated in slow-biased muscles, as well as candidates universally accessible across peak sets and located near slow myofiber genes up-regulated in slow-biased muscles. From these, six candidate CREs were prioritized for functional validation.

5.2.6 Luciferase reporter vector construction

Six candidate CRE DNA sequences were narrowed to regions < 500 bp based on phyloP conservation scores and screened for restriction enzyme cut sites using NEBcutter (v.3.0.17)⁴⁷⁴. For each CRE candidate, the human (hg19) and chimpanzee (panTro6) sequences were extracted from the UCSC Genome Browser, with sequence orientation adjusted to account for translocations. Differences in TF

binding motifs between human and chimpanzee sequences were analyzed using the JASPAR 2020 database⁵⁸⁹, and the R packages TFBSTools (v.1.44.0)⁵⁹⁰ and motifmatchr (v.1.28.0)⁵⁹¹. The twelve candidate CRE sequences were cloned upstream of the firefly luciferase reporter gene (*luc2*) promoter in the pGL4.23 [luc2/minP] vector (E8411, Promega) by Azenta Life Sciences. All reporter vector constructs were verified by Sanger sequencing⁴⁷⁵. Candidate CRE sequences, nearby gene functions, conservation scores, and restriction enzyme cut sites are provided in **Supplementary Table S3**.

5.2.7 Cell culture

Previous studies investigating *cis*- and *trans*-regulatory mechanisms in human skeletal muscle have commonly used human skeletal muscle myoblast (HSMM) cell lines for functional validation in undifferentiated myoblasts and differentiated myotubes^{592–599}. HSMM cells (Lonza, CC-2580) were cultured in SkBM-2 Basal Medium (Lonza, CC-3246) supplemented with GA-100, hEGF, Dexamethasone, fetal bovine serum, and L-Glutamine from the SkGM-2 SingleQuots Kit (Lonza, CC-3244) on 0.1% gelatin-coated cultureware at 37 °C in a humidified atmosphere with 5% CO₂. Growth medium was replaced every 1-2 days, and myoblasts were subcultured upon reaching ~80% confluency. For transfection experiments, undifferentiated HSMM myoblasts were seeded into 0.1% gelatin-coated 96-well plates and cultured for 48 hours prior to transfection. Due to time and financial constraints, transfections were not performed in differentiated HSMM myotubes, despite this being the original intent.

5.2.8 Transfection and luciferase reporter assays

HSMM myoblasts were transfected in 96-well plates using Lipofectamine 3000 (Invitrogen) following the manufacturer's protocol. Five independent transfection experiments ($n = 5$) were conducted, each with at least six technical replicates per reporter vector construct. An empty pGL4.23 vector (E8411, Promega) was used to determine baseline *luc2* expression. All reporter vector constructs were co-transfected with a pGL4.74 [hRluc/TK] *Renilla* vector (E6921, Promega) to control for transfection efficiency. Cells were harvested 48 hours post-transfection and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (E1910, Promega) on a GloMax Navigator microplate luminometer (GM2010, Promega), following the manufacturer's instructions.

Luciferase activity was calculated as the ratio of firefly (*luc2*) to *Renilla* (*Rluc*) luminescence, averaged across the technical replicates. The mean *luc2/Rluc* ratio for each reporter vector construct was then normalized to the empty pGL4.23 control vector to confirm regulatory activity in HSMM myoblasts. Significant differences in normalized luciferase activity were assessed using an unpaired two-tailed Student's *t*-test, and Fisher's combined probability test was used to integrate p-values across the seven transfection experiments. Data are presented as mean $\pm$ SEM (standard error of the mean) and statistical

significance is indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

5.3 Results

5.3.1 *Evolutionarily conserved mammalian CREs identified from mouse muscle show accessibility in human muscle tissue.*

Previously, we identified candidate muscle-specific CREs ($n = 44,644$ peaks) regulating genes involved in muscle development and myofiber physiology using a mouse model (Chapter 4). These peaks were distilled into 19 non-overlapping ‘condition-specific’ peak sets, representing peaks unique to muscles of a specific time point and myofiber bias (E18.5 Slow, Adult Slow, E18.5 Fast and Adult Fast), peaks shared across all muscles of a given time point (E18.5 and Adult), anatomical region (Calf and Thigh), or myofiber bias (Slow and Fast), and peaks shared across all muscles (Universal) (**Table 5.1**).

To target evolutionarily conserved mammalian CREs (e.g., CREs active across mammalian species), mouse (mm10) peak coordinates were lifted over to the human (hg19) genome assembly. A total of 33,860 peaks (75.8%) mapped to orthologous human coordinates, consistent with the overall degree of human-mouse genome alignment^{600,601}. Peaks that successfully mapped to the human genome were significantly more conserved than those that failed (success: 0.269 ± 0.654 ; failed: 0.0423 ± 0.137 ; $t = -61.247$, $df = 39,109$, $p = 2.2e-16$; **Fig. 5.2a**), supporting previous observations that evolutionarily conserved CREs are under higher evolutionary constraint than recently evolved, lineage-specific CREs⁶⁰².

Genomic annotation revealed that peaks failing to map to the human genome were predominantly located in distal intergenic regions ($F_{1,36} = 58.97$, $p = 4.3e-9$; **Fig. 5.2b**), whereas successfully mapped peaks were enriched in promoters ($F_{1,36} = 20.02$, $p = 7.4e-5$), introns ($F_{1,36} = 5.411$, $p < 0.0258$), exons ($F_{1,36} = 17.11$, $p = 0.0002$), and untranslated regions ($F_{1,36} = 26.68$, $p = 9.05e-6$; **Fig. 5.2c**). Peaks failing to map to the human genome, specifically those located in distal intergenic regions, may represent lineage-specific CREs unique to mice or rodents that contribute to unique aspects of their muscle biology^{601,603}, or they may simply reflect lineage-specific sequence modifications (e.g., insertions, deletions, inversions) that prevent orthologous alignment^{579,604–607}.

Next, to identify CREs with potential activity in human skeletal muscle, peaks mapping to the human genome were intersected with open chromatin peak sets generated from embryonic and adult human tissues (**Supplementary Table S1**). Universal peaks showed the strongest overlap across unfiltered human tissue peak sets (**Appendix V**), suggesting that these shared ‘skeletal muscle CREs’ are less tissue-specific and more pleiotropic than expected, with regulatory activity extending beyond limb musculature. To better capture tissue-specific signals and reduce pleiotropic CREs, human tissue peak

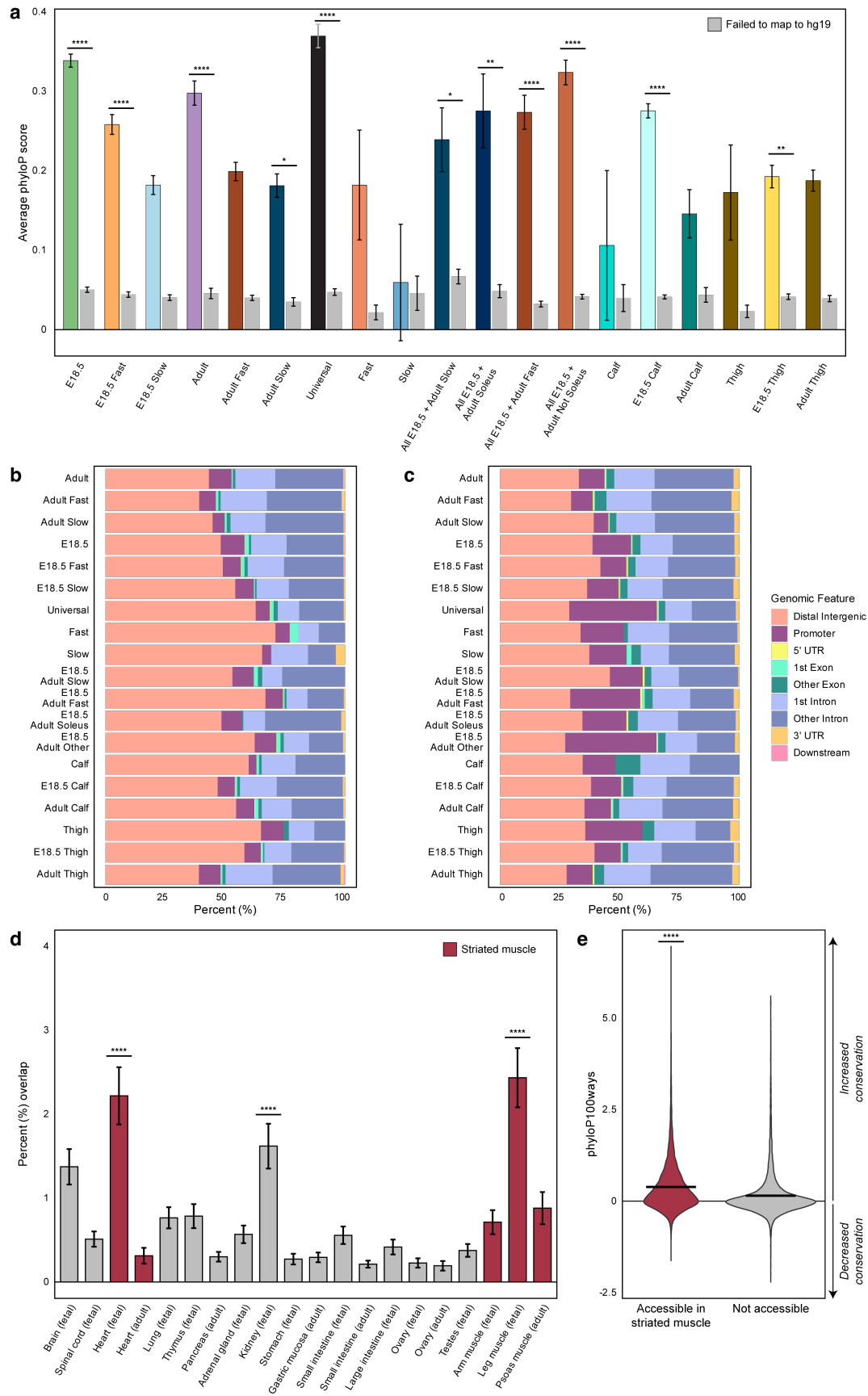
sets were filtered to retain peaks unique to each tissue and time point and intersected with condition-specific peaks. Unexpectedly, condition-specific peaks overlapped most strongly with fetal heart ($p = 9.2\text{e-}8$), fetal leg muscle ($p = 3.1\text{e-}7$), and fetal kidney ($p = 0.0004$) (**Fig. 5.2d**). Although the large size of these peak sets (fetal kidney, $n = 62,836$; fetal leg muscle, $n = 46,609$; fetal heart, $n = 37,314$) contributed to the significance ($S = 192.57$, $\rho = 0.856$, $p = 0.0000016$), this pattern may also reflect biological relationships, as cardiac and skeletal muscle share structural and functional properties⁶⁰⁸.

We adopted a stringent approach by focusing exclusively on evolutionarily conserved mammalian CREs overlapping a consensus “striated muscle” peak set generated from fetal heart, fetal leg, fetal arm, and adult psoas muscle ($n = 364,749$). Although this strategy likely excluded thousands of pleiotropic CREs important to muscle biology that are active across multiple tissues, it increased our confidence in identifying CREs most relevant to striated muscle, consistent with approaches taken for other tissues of interest^{412,413,609}. In total, 18,442 peaks (54.5%) overlapped with the striated muscle peak set, with no significant differences in overlap among peak sets (**Appendix W**). Peaks overlapping the striated muscle peak set were significantly more conserved than non-overlapping peaks (overlapping: 0.391 ± 0.690 ; non-overlapping: 0.150 ± 0.586 ; $W = 165846341$, $p = 2.2\text{e-}16$; **Fig. 5.2e**), suggesting that these functionally conserved, tissue-specific CREs are important for muscle development and biology. Collectively, these results highlight the utility of this conservative approach for identifying a subset of evolutionarily conserved mammalian CREs active in human striated muscle tissue.

Table 5.1. Number of mouse (mm10) condition-specific peaks that mapped to the human (hg19) genome assembly.

Peak Set	No. Peaks (mm10)	No. Peaks (hg19)	% Mapping to hg19
Universal	3,005	2,277	75.77
E18.5	7,564	6,960	92.01
Adult	1,717	1,614	94.00
Fast	231	101	43.72
E18.5 Fast	3,925	2,785	70.96
Adult Fast	3,641	2,821	77.48
E18.5 + Adult Fast	1,635	1,012	61.9
E18.5 + Adult Not Soleus	2,827	2,003	70.85
Slow	78	51	65.38
E18.5 Slow	3,805	2,458	64.60
Adult Slow	1,470	1,181	80.34
E18.5 + Adult Slow	393	257	65.39
E18.5 + Adult Soleus	332	190	57.23
Calf	97	29	29.90
E18.5 Calf	7,587	5,982	78.85
Adult Calf	588	344	58.50
Thigh	185	97	52.43
E18.5 Thigh	2,848	1,705	59.87
Adult Thigh	2,716	1,993	73.38

Fig. 5.2 (next page). Identification of evolutionarily conserved CREs with activity in human tissue. (a) Average per-bp phyloP conservation scores for fixed-length (400 bp) condition-specific peak sets. Asterisks indicate statistically significant differences between peaks that mapped to the human genome (colored bars) and those that failed (gray bars). (b,c) Proportion (%) of peaks overlapping annotated genomic features for peaks that (b) failed to map or (c) successfully mapped to the human genome. (d) Proportion (%) of condition-specific peaks overlapping each filtered human tissue peak set. Striated muscles are shown in raspberry. Asterisks denote significant differences in peak set overlap. (e) Average per-bp phyloP100ways conservation scores for fixed-length (400 bp) peaks overlapping (raspberry) and not overlapping (gray) a consensus human striated muscle dataset. Black lines indicate mean per-bp conservation scores. Positive scores reflect increased evolutionary constraint (slower-than-expected sequence change), whereas negative scores indicate decreased evolutionary constraint (faster-than-expected sequence change). Significant differences between peak sets are indicated by asterisks. $*p \leq 0.05$; $**p \leq 0.01$. $***p \leq 0.001$; $****p \leq 0.0001$.



5.3.2 Conserved mammalian CREs with activity in human striated muscle show enrichment for regions evolving faster in primates near muscle-associated genes.

To identify conserved mammalian CREs important to human evolution, peaks accessible in human striated muscle ($n = 18,442$) were intersected with evolutionary datasets containing human- and non-human primate-specific signals (genomic coordinates) of accelerated evolution (**Supplementary Table S2**)^{405,411,567–576}. Overall, 1,846 peaks (10.0%) overlapped regions under accelerated evolution in primates (humans plus non-human primate species), including 190 peaks (1%) overlapping human-specific signals of accelerated evolution (**Fig. 5.3a, Appendix X**). These 190 peaks represent conserved, striated muscle-specific CREs under accelerated evolution in the human lineage that may contribute to the fatigue-resistant properties of human skeletal muscle.

To determine whether these CREs were enriched with signals of accelerated evolution, and thus potentially important to the emergence of unique primate muscle phenotypes, we compared peaks overlapping evolutionary signals (human-specific, $n = 190$; primate, $n = 1,846$) against 1,000 matched random permutations. While peaks were not enriched for human-specific accelerated regions, Fisher's exact test revealed significant enrichment for non-human primate signals of accelerated evolution across several peak sets ($t = 2.78$, $p = 0.001$), including E18.5 ($p\text{-adj} < 2.7\text{e-}18$), E18.5 Fast ($p\text{-adj} < 2.6\text{e-}10$), E18.5 Slow ($p\text{-adj} < 5.1\text{e-}7$), Adult ($p\text{-adj} < 1.8\text{-}12$), Adult Fast ($p\text{-adj} < 2.7\text{e-}7$), Adult Slow ($p\text{-adj} < 0.007$), Universal ($p\text{-adj} < 6.6\text{ e-}63$), E18.5 Adult Fast ($p\text{-adj} < 4.9\text{e-}12$), E18.5 Adult Not Soleus ($p\text{-adj} < 2.6\text{e-}30$), E18.5 Calf ($p\text{-adj} < 8.7\text{e-}32$), E18.5 Thigh ($p\text{-adj} < 0.03$), and Adult Thigh ($p\text{-adj} < 0.002$) (**Fig. 5.3a**). This suggests that these conserved, striated muscle-specific mammalian CREs overlap regions of primate-specific accelerated evolution more than expected by chance and therefore may be important targets of primate muscle evolution more broadly.

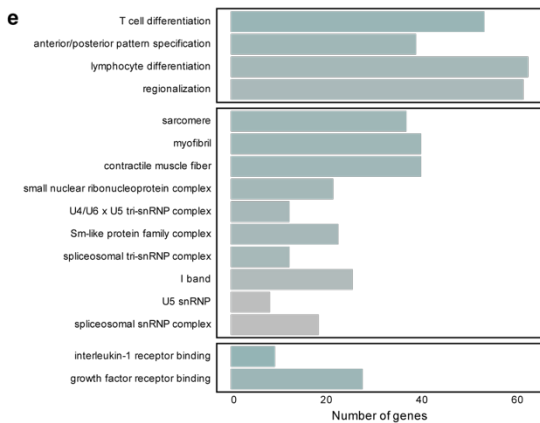
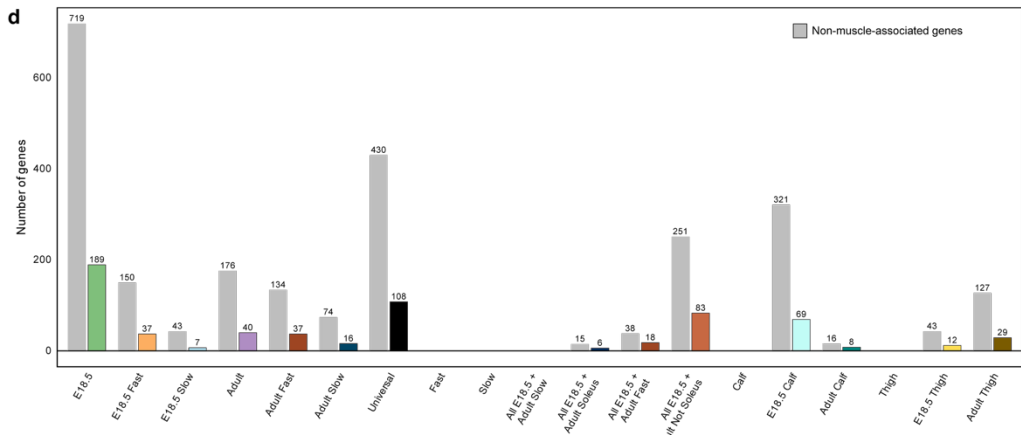
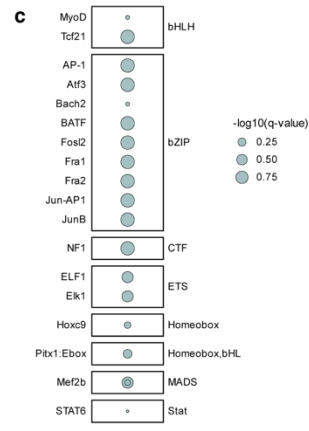
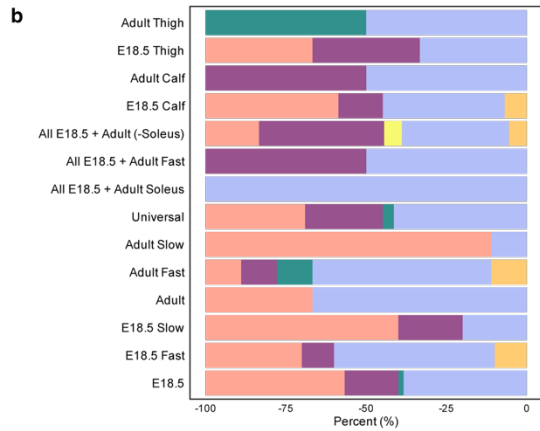
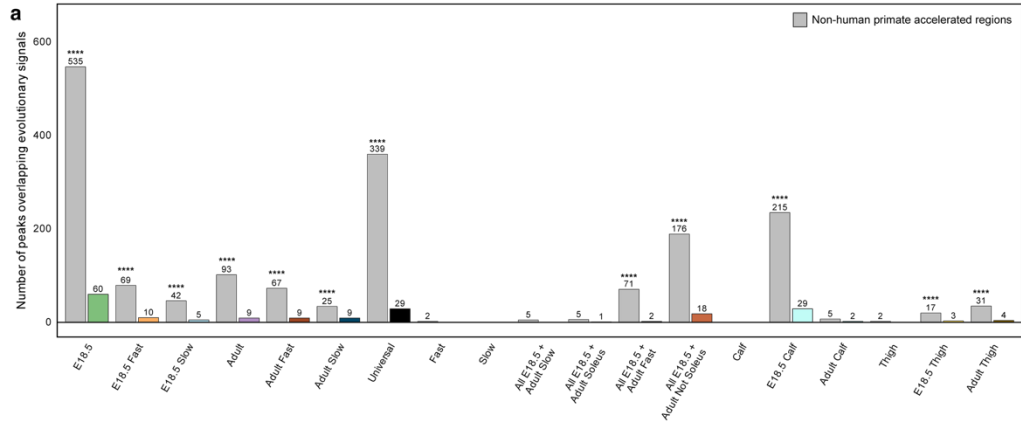
Peaks overlapping regions of human-specific accelerated evolution did not differ in genomic feature distribution, with most residing in promoters or in regions associated with tissue-specific CREs, such as distal intergenic regions and introns (**Fig. 5.3b**)^{610,611}. Transcription factor (TF) motif enrichment analysis was performed to assess whether peaks overlapping human-specific signals of accelerated evolution were enriched for TFs involved in myogenesis and myofiber differentiation. Peaks showed enrichment for bHLH and bZIP motifs, which regulate mesenchyme cell fate, neural development and cell differentiation (**Fig. 5.3c**)^{612–615}. As expected, motifs for MyoD and Mef2b, key regulators of muscle development, muscle cell fate, and myofiber differentiation^{224,616,617}, were identified. These results suggest that human-specific mutations within conserved mammalian promoters and tissue-specific CREs may have affected TF binding and gene expression during muscle development and myofiber differentiation, contributing to human's unique muscle physiology.

To identify CREs potentially regulating muscle-associated genes, genes located within $\pm 500,000$ bp

of each peak under human-specific accelerated evolution ($n = 190$) were assessed for GO function. Of 3,196 genes located within $\pm 500,000$ bp of each peak, 17.3% ($n = 544$) were annotated to muscle-related GO terms, compared to only 9.1% of genes surrounding primate accelerated regions ($n = 38,034$) (**Appendix Y**). The strongest enrichment of muscle-associated genes occurred near Adult Calf (33.3%, $n = 8$), E18.5 Adult Fast (32.1%, $n = 18$), E18.5 Adult Soleus (28.6%, $n = 6$), and E18.5 Adult Not Soleus (24.9%, $n = 83$) peaks (**Fig. 5.3d**). GO analysis of the 3,196 genes located within $\pm 500,000$ bp of each peak further revealed enrichment for terms related to myofiber structure and developmental patterning, as well as KEGG pathways linked to cancer (**Fig. 5.3e**, **Supplementary Table S4**).

Peaks overlapping human-specific signals of accelerated evolution harbored both disease-associated (ClinVar) and common SNPs (**Fig. 5.3f**). However, common SNP enrichment was not significantly different from a random background and no ClinVar SNPs were associated with muscle disease. Closer inspection revealed that ClinVar SNP enrichment was driven by peak overlap with promoters and first exons. For example, all 620 SNPs associated with the E18.5 Calf peak set mapped to the promoter and first exon of *HOXB13*. Thus, no muscle disease-associated SNPs directly overlapped conserved mammalian CREs under accelerated evolution in humans.

Fig. 5.3 (next page). Characterization of muscle-associated peaks overlapping primate evolutionary datasets. (a) Total number of peaks from each condition-specific peak set overlapping human (colored bars) and primate (gray bars) regions of accelerated evolution. Asterisks denote significant enrichment for evolutionary signals relative to the random background. (b) Proportion (%) of peaks overlapping annotated genomic features for peaks overlapping regions of human-specific accelerated evolution. (c) Top 15 enriched transcription factor (TF) binding site motifs identified across peaks overlapping regions of human-specific accelerated evolution, grouped by TF family. (d) Total number of genes within $\pm 500,000$ bp of each human evolutionary dataset-overlapping peak set. Colored bars indicate genes associated with muscle-related terms, while gray bars indicate genes without such associations. (e) Gene Ontology (GO) enrichment analysis of Biological Process, Cellular Component, and Molecular Function terms for genes within $\pm 500,000$ bp of each peak set. (f) Number of disease-associated (ClinVar) and common SNPs residing within peaks overlapping primate evolutionary datasets. Asterisks denote significant enrichment relative to the random background. Empirical $*p \leq 0.05$; $**p \leq 0.01$. $***p \leq 0.001$; $****p \leq 0.0001$.



f

Peak Set	No. ClinVar SNPs	No. Common SNPs
E18.5	15	266
E18.5 Fast	0	24
E18.5 Slow	0	3
Adult	0	25
Adult Fast	5*	44
Adult Slow	0	26
Universal	38*	106
Fast	0	0
Slow	0	0
E18.5, Ad. Slow	0	0
E18.5, Ad. Soleus	0	1
E18.5, Ad. Fast	0	219
E18.5, Ad. Not Soleus	0	58
Calf	0	0
E18.5 Calf	620****	94
Adult Calf	0	5
Thigh	0	0
E18.5 Thigh	0	16
Adult Thigh	0	178

5.3.3 Nine candidate CREs significantly enhance luciferase activity in human muscle cells.

To select CRE candidates for functional validation, the genomic landscape of peaks residing within regions of human-specific accelerated evolution was examined using the UCSC Genome Browser. Priority was given to peaks overlapping signals of human-specific accelerated evolution harboring human-specific sequence change located within 100 kb of muscle-related genes. From this candidate list, six CREs were prioritized for functional validation: four within Universally-accessible regions near slow myofiber genes, one within a Slow-biased muscle-accessible region near a slow myofiber gene, and one within an Adult Slow-biased muscle-accessible region near a slow myofiber gene (**Supplementary Table S3**). For each candidate, the human- and chimpanzee-specific sequences were functionally validated using a dual luciferase reporter assay in a human muscle cell line. Across experiments, candidates Universal 2 Human ($\chi^2 = 15.22$, $df = 10$, $p = 0.124$), Universal 4 Chimpanzee ($\chi^2 = 10.77$, $df = 10$, $p = 0.376$), and Universal 4 Human ($\chi^2 = 12.61$, $df = 10$, $p = 0.247$) did not drive luciferase expression above the control vector, suggesting weak enhancer activity (**Appendix Z**). In contrast, nine candidates significantly increased luciferase activity relative to the control: Universal 1 Chimpanzee ($\chi^2 = 77.75$, $df = 10$, $p = 2.0e-11$), Universal 1 Human ($\chi^2 = 62.14$, $df = 10$, $p = 1.4e-9$), Universal 2 Chimpanzee ($\chi^2 = 31.65$, $df = 10$, $p = 0.0005$), Universal 3 Chimpanzee ($\chi^2 = 77.45$, $df = 10$, $p = 1.6e-12$), Universal 3 Human ($\chi^2 = 46.21$, $df = 10$, $p = 0.000001$), Slow Chimpanzee ($\chi^2 = 51.79$, $df = 10$, $p = 0.0000001$), Slow Human ($\chi^2 = 32.12$, $df = 10$, $p = 0.0004$), Adult Slow Chimpanzee ($\chi^2 = 19.48$, $df = 10$, $p = 0.03$), and Adult Slow Human ($\chi^2 = 33.58$, $df = 10$, $p = 0.0002$), consistent with enhancer activity (**Fig. 5.4**). However, species-specific variation within candidate CREs did not yield significant differences in luciferase activity *in vitro*. Thus, while several conserved mammalian enhancers were identified, additional experiments are required to validate these trends and clarify the direct effect of each candidate on its putative target gene.

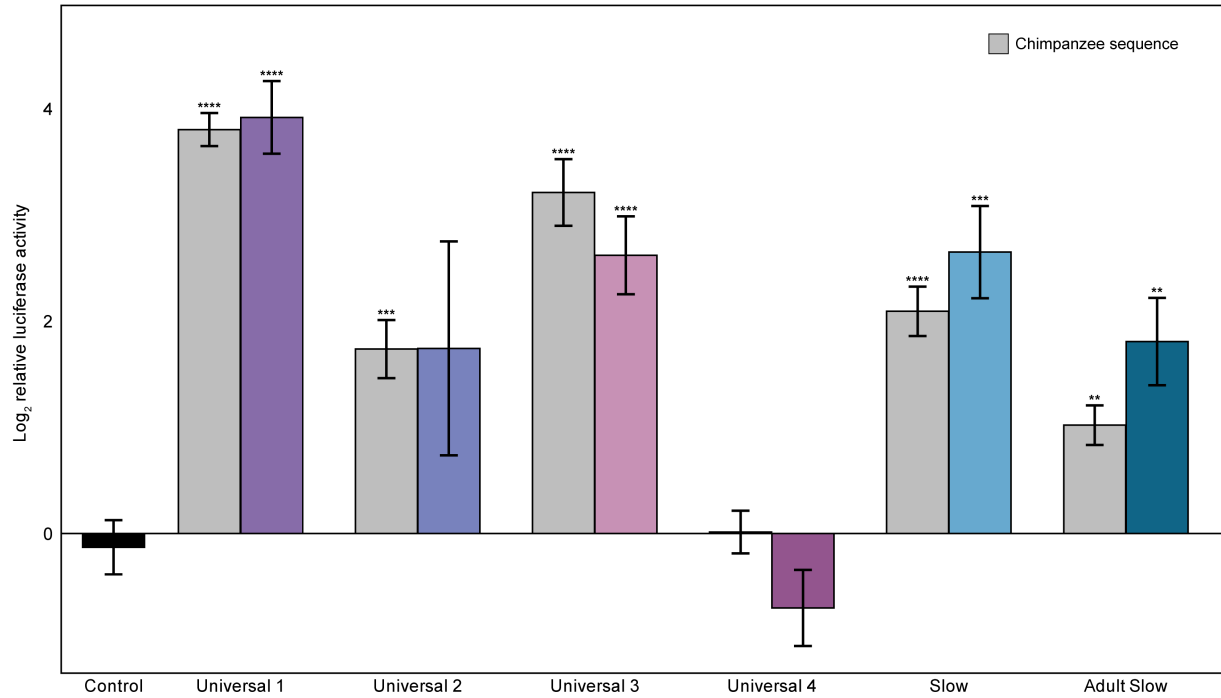


Fig. 5.4. Functional validation of human and chimpanzee candidate CRE sequences using a dual-luciferase reporter assay in HSM cells. Relative luciferase activity is shown for human (colored bars) and chimpanzee (gray bars) versions of six candidate CREs in human muscle cells (HSM). Positive log₂ fold change (log₂FC) values indicate enhanced luciferase activity, while negative values indicate suppressed luciferase activity relative to the empty pGL4.23 control vector. Data are presented as means $\pm$ SEM ($n = 7$). Asterisks denote statistically significant differences from the control vector for Experiment 5: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

5.4 Discussion

Our previous study (Chapter 4) identified thousands of open chromatin peaks (i.e., potential CREs) targeted to fast- and slow-biased mouse muscles at both embryonic and adult time points. Using stringent evolutionary genomics approaches, we refined this set to 190 candidate CREs that overlap regions of human-specific accelerated evolution and show evidence of activity in human muscle. These candidates represent evolutionarily conserved mammalian CREs that harbor human-specific sequence changes potentially under selection that may contribute to human muscle endurance. To test this possibility, we selected six candidates for functional validation, assaying the effects of human- and chimpanzee-specific sequence variation on gene expression in a human skeletal muscle cell line. Although nine candidates were capable of driving gene expression above control levels *in vitro*, none showed consistent species-specific differences in gene expression. Here we discuss these findings and future directions.

5.4.1 *Mouse models reveal evolutionarily conserved mammalian CREs important for human evolution*

Phenotypic divergence between species, including the evolution of unique traits, is largely attributed to species-specific differences in the spatiotemporal regulation of gene expression during development^{172,186,618–620}. These differences can arise from species-specific mutations within evolutionarily conserved CREs shared across species^{405,415,602,621–624}, or from the evolution of novel, lineage-specific CREs absent in other taxa^{625–628}. Indeed, the unique properties of the human brain has been attributed both to human-specific mutations within evolutionarily conserved CREs residing in regions of human-specific accelerated evolution^{407,408,629,630} and to the emergence of novel, human-specific CREs^{627,631,632}.

In this study, CRE candidates were generated using a mouse model, which biases discovery toward evolutionarily conserved mammalian CREs that may harbor human-specific sequence variation contributing to adaptations for long-distance bipedal locomotion (i.e., slow-biased muscles)⁴²³. Capturing recently evolved, human-specific CREs that a mouse-based approach cannot detect would require generating CRE candidates directly from human embryonic and adult muscles^{633–635}. However, given the paucity of late-stage embryonic primate samples, which are more relevant to myofiber differentiation, as well as the challenges surrounding collecting, accessing, and using human and non-human primate embryonic tissue, a mouse model remains the most pragmatic approach for identifying evolutionarily conserved CREs important to human evolution.

To identify evolutionarily conserved CREs relevant to human evolution, we focused on candidates overlapping regions of accelerated evolution specific to humans, non-human primates, and primate stem lineages. Overall, 1,846 peaks overlapped regions under accelerated evolution in primates, including 190 peaks overlapping human-specific accelerated regions. Although peak sets active in human striated muscle were not significantly enriched for human-specific signals, they harbored more primate accelerated regions than expected by chance, indicating that these conserved mammalian CREs are functionally important across primates, and not just humans, specifically. Notably, E18.5 peaks (representing potential muscle-specific CREs active during development) and Universal peaks (representing potential muscle-specific CREs active across the lifespan) were significantly enriched for signals of accelerated evolution. These findings suggest that conserved striated muscle-specific CREs that regulate embryonic muscle development and maintain myofiber identity may have been frequent targets of selection across diverse primate lineages, likely contributing to variation in muscle physiology and function, and to the diverse locomotor strategies employed by primates^{13,423,557}.

Of particular interest to human evolution are peaks overlapping human-specific signals of accelerated evolution, representing conserved, striated muscle-specific CREs that may contribute to the fatigue-resistant properties of human skeletal muscle. Human-specific sequence changes within CREs

active during embryonic development (e.g., E18.5, E18.5 Fast, E18.5 Calf) may have been selected for their effects on developmental gene expression and muscle biology. Mutations across multiple developmental CREs acting in concert could have established the regulatory environment necessary for the emergence of “human” traits such as fatigue-resistant, slow-biased muscles. Similarly, human-specific variation within Slow, E18.5 Slow, and Adult Slow-specific CREs may enhance pathways supporting slow myofibers and oxidative metabolism or repress those supporting fast myofibers and glycolytic metabolism, thereby promoting an adult muscle physiology adaptive for long-distance bipedal locomotion. However, without direct functional tests of these CREs during human muscle development *in vivo*, these conclusions remain speculative.

5.4.2 *Primate accelerated regions are compelling targets for future functional validation*

In addition to identifying CRE candidates under accelerated evolution in humans, this study also identified 1,656 CRE candidates overlapping regions of non-human primate- and primate stem lineage-specific accelerated evolution. Although “primate” and “ape” accelerated regions do not show an excess of human-specific sequence change, human-specific variants within these regions are still likely to have contributed to human evolution and the emergence of human-specific traits. Primate accelerated regions are also inherently interesting due to their potential importance for understanding primate evolution more broadly. While many studies have profiled the genomic landscape of primate accelerated regions (PARs)^{411,568,571,576,636}, none, to our knowledge, have functionally validated them *in vitro*, so their effects on gene expression remain unknown. Functional validation of conserved mammalian CREs overlapping non-human primate and stem primate lineage evolutionary signals would substantially advance the field and broaden its focus beyond humans.

Of particular interest are PARs located near muscle-associated genes, especially those under accelerated evolution in another slow-biased primate, the slow loris^{423,557}. These evolutionarily conserved CREs may have been repeatedly targeted by selection to promote primate muscle diversity and facilitate adaptation to distinct environments and locomotor strategies. However, the functional effects of species-specific mutations within these regions on gene expression and muscle myofiber content remain unknown. It is tempting to speculate that humans and slow lorises may share functionally equivalent mutations in conserved mammalian CREs regulating myofiber development and differentiation, thereby converging on a slow-biased muscle phenotype⁴²³. Future studies of skeletal muscle endurance should therefore investigate the non-coding genomes of other slow-biased species like sloths and slow lorises, and target regions of overlap.

5.4.3 *Candidate CREs overlapping human-specific accelerated regions are functional but do not drive species specific differences in gene expression*

Functional validation of human- and chimpanzee-specific sequence variation within six candidate CREs overlapping regions of human-specific accelerated evolution near muscle-associated genes revealed that nine candidates could significantly enhance gene expression in an undifferentiated human skeletal muscle cell line. However, species-specific mutations within these CREs did not produce significant differences in gene expression. This may be due to the undifferentiated state of HSMC myoblasts, as these CREs may require the regulatory background present in differentiated myotubes to display species-specific effects. Similarly, chimpanzee-specific variation within these candidates may require the regulatory environment of differentiated chimpanzee muscle cells to exhibit their full regulatory potential. Thus, while these experiments demonstrate that these candidates function as enhancers in undifferentiated human myoblasts, the full impact of species-specific variation on TF binding and target gene expression may depend on the native species-specific regulatory environment, which cannot be fully captured in cell culture or even in humanized mouse models^{637–639}.

Further experiments in differentiated human and chimpanzee skeletal muscle cell lines are needed to resolve these patterns, as enhancer activity is highly context dependent, influenced by the expression of transcription factors and post-transcriptional and post-translational regulators (e.g., microRNAs, lncRNAs) present at specific time points or in specific tissues^{640–643}. This consideration is especially important for the ‘Universal’ CREs, which are accessible in all muscles at both time points. Their activity likely depends on a variety of context-specific environmental cues and may function as both enhancers and silencers. It is also possible that species-specific variation within CREs under human-specific accelerated evolution has little to no effect on gene expression⁶⁴⁴, potentially due to redundant shadow enhancers⁶⁴⁵ located elsewhere in the genome. Therefore, functional validation of the remaining 184 candidate CREs is critical to extending this investigation into the role of functionally conserved, muscle specific CREs in human muscle evolution.

Despite these complexities, general trends in gene expression emerge across candidate CREs. The Universal 1 and Universal 3 drive the highest activity in vitro, consistent with strong enhancer function. Universal 1 is associated with *CTBP2*, which suppresses *PPAR α* , a key regulator of mitochondrial biogenesis^{646,647}. Elevated *CTBP2* activity may therefore promote fast myofiber pathways by inhibiting slow myofiber activators. Universal 3 is linked to *TCF7L2*, a TF that interacts with numerous fast- and slow myofiber-associated genes, including *EPAS1*, *MYC*, *MYLK2*, *MYO6*, and *PPARGC1A*^{136,648,649}. Across experiments, the humanized version of Universal 3 shows reduced activity, suggesting that human-specific mutations may suppress *TCF7L2* expression, leading to either fast- and slow-biased muscle phenotypes. Universal 4 appears to act as a basal enhancer associated with *PPARD*/ δ , which

regulates myofiber phenotypes, lipid metabolism and muscle endurance^{650–652}. The lower activity of the humanized version suggests that human-specific sequence variation (including the hCONDEL) may reduce *PPARD/δ* expression and promote fast myofiber pathways. In contrast, the humanized version of Adult Slow trends toward higher activity *in vitro*, raising the possibility that human-specific sequence variation enhances *MEF2C* expression in adult muscles, promoting slow myofiber pathways and leading to slow-biased muscles^{224,616,653}.

Here, we used a mouse model to identify evolutionarily conserved mammalian CREs harboring species-specific variation that can enhance or suppress gene activity *in vitro*. Our stringent evolutionary genomics approach allowed us to identify 190 candidate CREs active in human striated muscle that show evidence of accelerated evolution in humans, potentially contributing to the fatigue-resistant properties of human skeletal muscle that optimize them for long-distance bipedal locomotion. We validated human- and chimpanzee-specific variation on gene expression for six candidates, and while nine candidates functioned as enhancers *in vitro*, none displayed significant species-specific differences in gene expression, though interesting trends were observed. Further experiments in differentiated human and chimpanzee skeletal muscle cell lines are needed to capture the native species-specific regulatory environments required to resolve complex regulatory dynamics not addressed here. These findings demonstrate that aspects of human skeletal muscle physiology, though undetectable in the fossil record, can be studied through an evolutionary lens using comparative genomics.

6 CONCLUSION

Among primates, humans are unique in being habitual bipeds and are the only extant species capable of long-distance, long duration (i.e., endurance) running¹. This endurance capacity is supported in part by the high proportion of fatigue-resistant slow myofibers in hind limb muscles, which enhance muscular endurance and locomotor performance^{150–152,424}. Consequently, skeletal muscle myofiber content (i.e., the proportion of slow myofibers within each muscle) was likely a target of selection, with mutations in non-coding *cis*-regulatory elements (CREs) that promote slow myofiber gene pathways becoming fixed in the hominin lineage^{172,654}. The goal of this dissertation was to identify conserved, muscle-specific CREs overlapping regions of human-specific accelerated evolution associated with myofiber type, and to test whether species-specific variation within these CREs altered gene expression *in vitro*. We hypothesize that selection for more efficient bipedal locomotion drove the fixation of human-specific substitutions within these CREs, resulting in a higher proportion of fatigue-resistant slow myofibers in the human hind limb.

Chapters 2 and 3 placed humans within a broader mammalian context, asking how “unique” human muscles truly are and whether human behavior (e.g., exercise) could make them appear more “chimpanzee-like” (i.e., fast myofiber-biased). As expected, we found that the greater proportion of slow myofibers in human muscles cannot be explained by body size alone. By combining novel histochemical data generated from a large African ape (*Gorilla gorilla*) with publicly available myofiber composition data from over 150 mammalian species, we show that larger-bodied species generally exhibit higher proportions of slow myofibers, consistent with previous observations^{131,655}, yet humans fall into a small subset of mammals with far more slow myofibers than predicted by size. This subset includes sloths and slow lorises, whose locomotor strategies, like that of humans, benefit from the fatigue-resistant properties of slow myofibers, enabling them to sustain tonic contractions during bridging and cryptic climbing. These parallels raise intriguing questions about the genetic basis of slow-biased muscles in these species. One possibility is that humans, sloths, and slow lorises converged upon similar CRE mutations that promote slow myofiber pathways or suppress fast myofiber pathways. However, comparative analyses are currently limited as available reference genomes for sloths (GCA_000164785.2/C_hoffmanni-2.0.1, GCA_015220235.1/mChoDid1^{656,657}) and slow lorises (GCA_023898255.1/ASM2389825v1, GCA_023783135.1/ASM2378313v1, GCA_027406575.1/mNycCou1⁶⁵⁷) are lower coverage than the human genome and biased toward protein-coding regions, leaving non-coding CREs poorly characterized. Thus, further investigations into the convergent evolution of slow-biased muscles will require the generation of more complete sloth and slow loris genomes.

Data from **Chapter 2** further indicate that muscle myofiber content is a trait that distinguishes

humans from other African apes, likely established during development through gene expression programs maintained throughout life. Normal human activity (e.g., running, weightlifting) does not induce shifts large enough to overcome the difference in slow myofiber content between humans and chimpanzees. Indeed, the most dramatic shifts occur under extreme conditions such as spaceflight and spinal cord injury, which are either irrelevant to early hominins or represent severe injuries unlikely to be favored as mechanisms of evolutionary change. These examples demonstrate that muscle pathways *can* be altered under extraordinary circumstances to generate significant differences in muscle myofiber composition. However, they also highlight that typical adult human behavior cannot overcome intrinsic species-specific differences in muscle myofiber composition, which are genetically determined. In short, humans cannot exercise their way to “chimpanzee super-strength.”

Given the genetic basis of muscle myofiber composition, **Chapter 4** focuses on identifying the CREs that regulate myofiber type as a first step toward understanding human muscle evolution. Using a mouse model, we characterized the regulatory landscape of late developmental (E18.5) and adult hind limb muscles from two anatomical compartments (calf and thigh) that differ in slow myofiber content. We observed distinct patterns of gene expression and chromatin accessibility that reflect the biological processes active at each time point. At E18.5, regulatory signatures were primarily driven by anatomical compartment (calf v thigh), whereas in adults they were shaped by muscle physiology (fast v slow) and identity (soleus v gastrocnemius v vastus intermedius v vastus lateralis). Few genes maintained consistent expression across the lifespan, highlighting both the importance of housekeeping genes for cell identity and the role of time- and context-dependent gene expression in muscle development and differentiation. Surveying muscle-specific CREs unique to fast- and slow-biased muscles ($n = 13,150$), we prioritized 12 candidates for functional validation *in vitro*. Of these, nine acted as enhancers and three as silencers, confirming that our approach successfully identified active CREs in mouse skeletal muscle. However, we were unable to definitively link the activity of these candidates to specific target genes, and time and funding constraints prevented testing in differentiated C2C12 myotubes. As a result, the regulatory targets of these CREs and their influence on gene expression in a context more representative of adult muscle remain unresolved. Future *in vivo* mouse studies will be essential to define their target genes and quantify their effects on phenotype^{639,658}.

In **Chapter 5**, we hypothesized that some of the CREs identified in Chapter 4 would be functional in humans and contain human-specific variants that promote slow myofiber pathways, contributing to slow-biased muscles. To identify evolutionarily conserved mammalian CREs active in human muscle and potentially important for human evolution, we intersected mouse-derived CRE candidates with publicly available human muscle open chromatin datasets and regions of human-specific accelerated evolution. These stringent filtering steps yielded 190 functionally conserved, muscle-specific CREs that may harbor

human-specific sequence changes contributing to the unique fatigue-resistant properties of human skeletal muscle. Due to time and funding constraints, we prioritized six candidates near slow myofiber-associated genes and tested the effects of human- and chimpanzee-specific variants on gene expression *in vitro*. Of these, nine acted as enhancers, although none displayed significant species-specific effects on gene expression, despite some suggestive trends. Future experiments using differentiated human and chimpanzee skeletal muscle cell lines will be critical for capturing native species-specific regulatory environments and resolving complex regulatory dynamics not addressed here. However, to our knowledge, no commercial chimpanzee skeletal muscle cell line currently exists, although generating myotubes from induced pluripotent stem cells has been done for macaques and humans^{659,660}. Nonetheless, we demonstrated that evolutionarily conserved mammalian CREs overlapping regions of human-specific accelerated evolution can drive gene expression *in vitro*, highlighting the utility of this approach for identifying CREs potentially co-opted in the human lineage to shape muscle endurance.

Overall, our studies demonstrate that: (1) human muscles are unique among African apes but resemble those of slow arboreal climbers such as sloths and slow lorises, whose muscles are also slow-biased; (2) typical adult human behavior cannot shift muscle myofiber composition to match that of our closest living relatives, the African apes; (3) thousands of CREs unique to fast- and slow-biased muscles, including 9 enhancers and three silencers, may contribute to muscle myofiber composition; and (4) natural selection may have repeatedly targeted evolutionarily conserved CREs important for muscle development and identity, promoting muscle diversity across primates and contributing to the fatigue-resistant properties of human skeletal muscle. While the phenotypic effects of these CREs remain speculative, our results suggest that evolutionarily conserved mammalian CREs have undergone accelerated evolution not only in humans, but in other primate lineages as well. This implies that these CREs have played important roles in diverse aspects of muscle biology and have likely been co-opted multiple times to help species adapt to their unique environments and modes of locomotion. To date, no studies have functionally validated non-human primate accelerated regions, despite their potential significance for understanding primate evolution. Functional validation of conserved mammalian CREs overlapping non-human primate evolutionary signals would substantially advance the field and broaden its focus beyond humans. Of particular interest are PARs near muscle-associated genes, especially those showing accelerated evolution in another slow-biased primate, the slow loris^{423,557}. Species-specific mutations within these regions presumably alter gene expression and phenotype, yet their functional effects remain unknown. Key questions for future research include: Do human and slow loris accelerated regions appear near the same muscle-associated genes? Do humans and slow lorises share the same mutations within species-specific regions of accelerated evolution? Do these species-specific accelerated regions reflect alternative regulatory pathways that converge on a slow-biased phenotype?

These studies generated thousands of CRE candidates, including those relevant to mouse muscle development ($n = 44,644$), evolutionarily conserved striated-muscle specific CREs relevant to primate evolution ($n = 1,656$) and human evolution ($n = 190$), as well as many pleiotropic CREs active in skeletal muscle that did not meet our stringent filtering criteria. Validating all candidates would require resources beyond the scope of a dissertation. However, if time and funding were not a constraint, massively parallel reporter assays in differentiated human and non-human primate muscle cell lines could be used to test the function of all candidates *in vitro*, enabling rapid identification of functional CREs for further testing with species-specific variants⁶⁶¹. Similarly, future studies generating CRE candidates directly from human embryonic and adult muscles will be critical for identifying recently-evolved, human-specific CREs that are missed when relying on a mouse model^{633–635}. However, given the scarcity of late-stage embryonic primate samples, as well as the challenges surrounding collecting, accessing, and using human and non-human primate embryonic tissue, a mouse model remains the most pragmatic approach.

7 APPENDICES

7.1 Appendix A. Studies reporting mammalian skeletal muscle myofiber content.

Order	Family	Scientific name	Common name	No. of individuals	References
Artiodactyla	Balaenopteridae	<i>Balaenoptera physalus</i>	Fin whale	8	Hochachka and Foreman, 1993; Rivero, 2018
	Bovidae	<i>Antidorcas marsupialis</i>	Springbok	26	Curry et al., 2012; North and Hoffman, 2017, 2015
		<i>Bos taurus</i>	Cattle (domestic)	89	Brandstetter et al., 1998; Gotoh, 2003; Kadim et al., 2009; Mashima et al., 2019; Song et al., 2020; Suzuki and Tamate, 1974
		<i>Bos taurus indicus</i>	Zebu	25	Hena et al., 2018
		<i>Bubalus bubalis</i>	Mediterranean buffalo	28	Francisco et al., 2011
		<i>Connochaetes gnou</i>	Black wildebeest	13	Kohn et al., 2007, 2011b
		<i>Connochaetes taurinus</i>	Blue wildebeest	3	Kohn et al., 2007
		<i>Damaliscus pygargus phillipsi</i>	Blesbok	16	Kohn, 2014; Kohn et al., 2007
		<i>Ovis aries</i>	Sheep (domestic)	35	Konno and Watanabe, 2012; Suzuki, 1995, 1991, 1972, 1971a, 1971b; Suzuki and Tamate, 1988; Watanabe and Suzuki, 1999
		<i>Redunca fulvorufula</i>	Mountain reedbuck	13	Kohn, 2014
		<i>Tragelaphus strepsiceros</i>	Greater kudu	7	Kohn, 2014; Kohn et al., 2007
	Camelidae	<i>Camelus dromedarius</i>	Dromedary camel	63	Hena et al., 2018; Kadim et al., 2009; Wenfang et al., 2019
		<i>Camelus ferus</i>	Wild Bactrian camel	18	Wenfang et al., 2019
		<i>Llama glama</i>	Llama	31	Graziotti et al., 2012, 2004, 2001
	Cervidae	<i>Capreolus capreolus</i>	Roe deer	13	Żochowska-Kujawska et al., 2009, 2007
		<i>Cervus elaphus</i>	Red deer	29	Żochowska-Kujawska, 2016; Żochowska-Kujawska et al., 2009
		<i>Dama dama</i>	European fallow deer	40	Curry et al., 2012; Żochowska-Kujawska et al., 2019, 2009
		<i>Rangifer tarandus</i>	Reindeer	27	Essén-Gustavsson and Rehbinder, 1985; Pösö et al., 1996
		<i>Rangifer tarandus platyrhynchus</i>	Svalbard reindeer	12	Kiessling and Kiessling, 1984
	Delphinidae	<i>Sagmatias obliquidens</i>	Pacific white-sided dolphin	1	Ponganis and Pierce, 1978
		<i>Tursiops truncatus</i>	Common bottlenose dolphin	6	Bello et al., 1985; Kielhorn et al., 2013

Carnivora	Giraffidae	<i>Giraffa camelopardalis</i>	Northern giraffe	1	Roy et al., 1988
	Kogiidae	<i>Kogia breviceps</i>	Pygmy sperm whale	5	Kielhorn et al., 2013
	Monodontidae	<i>Monodon monoceros</i>	Narwhal	3	Williams et al., 2011
	Suidae	<i>Sus scrofa domesticus</i>	Pig (domestic)	809	Beecher et al., 1965; Essén-Gustavsson and Fjellkner-Modig, 1985; Karlsson et al., 1993; Lefaucheur et al., 2002; Rehfeldt et al., 2008; Ryu et al., 2008; Song et al., 2020; Suzuki et al., 1999; Wojtysiak et al., 2016
		<i>Sus scrofa scrofa</i>	Wild boar	93	Rehfeldt et al., 2008; Skewes et al., 2014; Źochowska et al., 2005; Źochowska-Kujawska et al., 2012, 2009, 2007, 2006
	Ailuridae	<i>Ailurus fulgens</i>	Red panda	1	Rowlerson et al., 1983
	Canidae	<i>Canis lupus familiaris</i>	Dog (domestic)	90*	Acevedo and Rivero, 2006; Armstrong et al., 1982; Braund et al., 1982; de A. Braga et al., 2016; Maxwell et al., 1977; Rowlerson et al., 1983; Toniolo et al., 2007
		<i>Vulpes vulpes</i>	Red fox	3	Rowlerson et al., 1983
		<i>Acinonyx jubatus</i>	Cheetah	6	Goto et al., 2013b; Hyatt et al., 2010; Williams et al., 1997
	Felidae	<i>Caracal caracal</i>	Caracal	1	Kohn et al., 2011a
		<i>Felis catus</i>	Cat (domestic)	108*	Ariano et al., 1973; Braund et al., 1995; Burke, 1967; Burke et al., 1974, 1973; Carlson, 1978; Chanaud et al., 1991; Collatos et al., 1977; Donselaar et al., 1987; Edström and Lindquist, 1973; English and Letbetter, 1982; Gonyea and Ericson, 1977; Hyatt et al., 2010; Maxwell et al., 1977; Richmond and Abrahams, 1975; Rowlerson et al., 1983; Talmadge et al., 1996; Wada et al., 1994
		<i>Panthera leo</i>	Lion	2	Kohn et al., 2011a
		<i>Panthera pardus melas</i>	Javan leopard	1	Rowlerson et al., 1983
		<i>Panthera tigris</i>	Tiger	2	Hyatt et al., 2010
	Mephitidae	<i>Mephitis mephitis</i>	Striped skunk	3	van de Graaff et al., 1977
	Mustelidae	<i>Enhydra lutris</i>	Sea otter	1	Ponganis and Pierce, 1978
		<i>Martes foina</i>	Stone marten	1	Rowlerson et al., 1983
		<i>Mustela putorius furo</i>	Domestic ferret	4	Moritz et al., 2007; Rowlerson et al., 1983
	Otariidae	<i>Arctocephalus pusillus doriferus</i>	Australian fur seal	16	LaRosa et al., 2012
		<i>Arctophoca gazella</i>	Antarctic fur seal	2	Reed et al., 1994
	Phocidae	<i>Halichoerus grypus</i>	Gray seal	7	Reed et al., 1994
		<i>Leptonychotes weddelli</i>	Weddell seal	13	Kanatous et al., 2002; Watson et al., 2003
		<i>Phoca vitulina</i>	Harbor seal	20	Hochachka and Foreman III, 1993; Reed et al., 1994
	Ursidae	<i>Ursus americanus</i>	American black bear	33	Riley et al., 2018; Rourke et al., 2006; Tinker et al., 1998

		<i>Ursus arctos</i>	Grizzly bear	12	Hershey et al., 2008; Salmov et al., 2015; Smerdu et al., 2009
		<i>Ursus maritimus</i>	Polar bear	5	Whiteman et al., 2017
		<i>Ursus thibetanus ussuricus</i>	Himalayan black bear	1	Salmov et al., 2015
Chiroptera	Craseonycteridae	<i>Craseonycteris thonglongyai</i>	Bumblebee bat	*	Jürgens, 2002
	Molossidae	<i>Tadarida brasiliensis cynocephala</i>	Brazilian free-tailed bat	8	Foehring and Hermanson, 1984
	Phyllostomidae	<i>Artibeus jamaicensis</i>	Jamaican fruit bat	6	Hermanson and Foehring, 1988
		<i>Artibeus lituratus</i>	Great fruit-eating bat	4	Hermanson et al., 1998
		<i>Carollia perspicillata</i>	Seba's short-tailed bat	4	Hermanson et al., 1998
		<i>Desmodus rotundus</i>	Common vampire bat	6	Hermanson et al., 1993
		<i>Diaemus youngi</i>	White-winged vampire bat	2	Hermanson et al., 1998
		<i>Glossophaga soricina</i>	Pallas's long-tongued bat	3	Hermanson et al., 1998
		<i>Phyllostomus hastatus</i>	Greater spear-nosed bat	3	Hermanson et al., 1998
		<i>Platyrrhinus helleri</i>	Heller's broad-nosed bat	3	Hermanson et al., 1998
	Pteropodidae	<i>Rousettus aegyptiacus</i>	Egyptian fruit bat	1	Luziga et al., 2021
	Rhinolophidae	<i>Rhinolophus cornutus</i>	Little Japanese horseshoe bat	3	Luziga et al., 2021
		<i>Rhinolophus ferrumequinum</i>	Greater horseshoe bat	3	Luziga et al., 2021
		<i>Rhinolophus mehelyi</i>	Mehely's horseshoe bat	8	Cebesoy, 2009
	Vespertilionidae	<i>Myotis myotis</i>	Greater mouse-eared bat	*	Cebesoy and Ayvali, 2003
		<i>Myotis lucifugus</i>	Little brown bat	53	Armstrong et al., 1977; Brigham et al., 1990; Hermanson et al., 1991
Cingulata	Dasypodidae	<i>Dasypus novemcinctus</i>	Nine-banded armadillo	4	Olson et al., 2016
Dasyuromorphia	Dasyuridae	<i>Antechinus flavipes</i>	Yellow-footed antechinus	4	Zhong et al., 2010
Didelphimorphia	Didelphidae	<i>Caluromys derbianus</i>	Derby's woolly opossum	6	Rupert et al., 2014
		<i>Didelphis virginiana</i>	Virginia opossum	19	Hansen et al., 1987; Hazimihalis et al., 2013; Peters et al., 1984
		<i>Monodelphis domestica</i>	Gray short-tailed opossum	5	Rupert et al., 2014
Diprotodontia	Macropodidae	<i>Macropus eugenii</i>	Tammar wallaby	1	Zhong et al., 2008
		<i>Macropus fuliginosus melanops</i>	Western grey kangaroo	2	Dennington and Baldwin, 1988
		<i>Macropus giganteus</i>	Eastern grey kangaroo	6	Spiegel et al., 2002; Zhong et al., 2008
		<i>Macropus parma</i>	Parma wallaby	3	Dennington and Baldwin, 1988
		<i>Osphranter rufus</i>	Red kangaroo	1	Zhong et al., 2008

Eulipotyphla	Potoroidae	<i>Setonix brachyurus</i>	Quokka	2	Casinos et al., 2016
		<i>Wallabia bicolor</i>	Swamp wallaby	1	Zhong et al., 2008
	Soricidae	<i>Bettongia penicillata ogilbyi</i>	Brush-tailed bettong	1	Zhong et al., 2008
		<i>Chimarrogale platycephalus</i>	Japanese water shrew	1	Ichikawa et al., 2019
		<i>Crocidura dsinezumi</i>	Dsinezumi shrew	1	Ichikawa et al., 2019
		<i>Crocidura russula</i>	Greater white-toothed shrew	6	Peters et al., 1999
		<i>Sorex araneus</i>	Eurasian shrew	37	Savolainen and Vornanen, 1995a, 1995b
		<i>Suncus etruscus</i>	Etruscan shrew	24	Jürgens, 2002; Peters et al., 1999
		<i>Suncus murinus</i>	Asian house shrew	7	Ichikawa et al., 2019; Suzuki, 1990
		Talpidae	<i>Mogera imaizumii</i>	Small Japanese mole	1
	<i>Mogera wogura</i>		Japanese mole	2	Ichikawa et al., 2019
<i>Scapanus latimanus</i>	Broad-footed mole		2	Goldstein, 1971	
<i>Urotrichus talpoides</i>	Japanese shrew mole		2	Ichikawa et al., 2019	
Lagomorpha	Leporidae	<i>Oryctolagus cuniculus domesticus</i>	Rabbit (domestic)	28*	Aigner et al., 1993; Fuentes et al., 1998; Gray and Renkin, 1978; Hämäläinen and Pette, 1993; Mabuchi et al., 1982; McFadden et al., 1984; Peter et al., 1972; Rab et al., 2000
Perissodactyla	Ochotonidae	<i>Ochotona rufescens</i>	Afghan pika	2	Schilling, 2005
	Equidae	<i>Equus africanus asinus</i>	Donkey	5	Snow and Guy, 1980
		<i>Equus ferus caballus</i>	Horse (domestic)	193	Eizema et al., 2005, 2003; Enríquez et al., 2015; Gellman et al., 2002; Grotmol et al., 2002; Kawai et al., 2009; Lindholm and Piehl, 1974; Raub et al., 1985; Rivero et al., 1999, 1996; Ronéus et al., 1992; Snow and Guy, 1980
Pilosa		<i>Equus ferus przewalskii</i>	Przewalski's wild horse	3	Bao et al., 2020
	Bradypodidae	<i>Bradypus variegatus</i>	Brown-throated sloth	5	Spainhower et al., 2021, 2018
	Choloepodidae	<i>Choloepus hoffmanni</i>	Hoffmann's two-toed sloth	4	Spainhower et al., 2021, 2018
			Sloth	*	Toole and Bullock, 1973
Primates	Aotidae	<i>Aotus azarae</i>	Azara's night monkey	*	Omstead et al., 2021, 2020
		<i>Aotus trivirgatus</i>	Northern night monkey	6	Plaghki et al., 1981
	Callitrichidae	<i>Callithrix geoffroyi</i>	White-headed marmoset	1	Potau et al., 2018
		<i>Callithrix jacchus</i>	Common marmoset	5	Plas et al., 2015; Potau et al., 2018
		<i>Callithrix penicillata</i>	Black-tufted marmoset	1	Rowlerson et al., 1983

	<i>Cebuella pygmaea</i>	Western pygmy marmoset	1	Potau et al., 2018
	<i>Leontopithecus chrysomelas</i>	Golden-headed lion tamarin	1	Potau et al., 2018
	<i>Saguinus imperator</i>	Emperor tamarin	1	Potau et al., 2018
	<i>Saguinus labiatus</i>	White-lipped tamarin	1	Muchlinski et al., 2018
	<i>Saguinus oedipus</i>	Cotton-top tamarin	3	Potau et al., 2018; Schmidt and Schilling, 2007
Cebidae	<i>Saimiri sciureus</i>	Common squirrel monkey	6	Potau et al., 2018; Rowleson et al., 1983; Schmidt and Schilling, 2007
	<i>Sapajus apella</i>	Tufted capuchin	1*	Muchlinski et al., 2018; Omstead et al., 2021, 2020
Cercopithecidae	<i>Cercocebus atys lunulatus</i>	White-crowned mangabey	1	Potau et al., 2018
	<i>Cercopithecus hamlyni</i>	Owl-faced monkey	2	Jouffroy and Médina, 1996; Muchlinski et al., 2018; Omstead et al., 2021
	<i>Chlorocebus sabaeus</i>	Green vervet monkey	8	Feng et al., 2012
	<i>Chlorocebus aethiops</i>	Grivet	5	Huq, 2013; Potau et al., 2011
	<i>Colobus guereza</i>	Mantled guereza	3	Potau et al., 2011; Muchlinski et al., 2018
	<i>Erythrocebus patas</i>	Patas monkey	5	Huq, 2013; Potau et al., 2011
	<i>Lophocebus albigena</i>	Gray-cheeked mangabey	1	Jouffroy and Medina, 1996
	<i>Macaca fascicularis</i>	Crab-eating macaque	16	Acosta and Roy, 1987; Muchlinski et al., 2018; Potau et al., 2018, 2011; Rowleson et al., 1983; Roy et al., 1984
	<i>Macaca fuscata</i>	Japanese macaque	13	Ito, 1998; Kimura, 2002; Suzuki and Hayama, 1994, 1991
	<i>Macaca mulatta</i>	Rhesus macaque	43	Cordonnier et al., 1995; Fitts et al., 1998; Ford et al., 1986; Jouffroy et al., 1999; Jouffroy and Médina, 1996; McIntosh et al., 1986; Richmond et al., 2001; Roy et al., 1991; Singh et al., 2002; Sokoloff et al., 2007
	<i>Macaca silenus</i>	Lion-tailed macaque	2	Potau et al., 2011
	<i>Mandrillus sphinx</i>	Mandrill	1	Potau et al., 2011
	<i>Miopithecus talapoin</i>	Angolan talapoin	5	Muchlinski et al., 2018; Potau et al., 2018, 2011
	<i>Papio anubis</i>	Anubis baboon	2	Kimura, 2002
	<i>Papio hamadryas</i>	Hamadryas baboon	2	Kimura, 2002
	<i>Papio ursinus ursinus</i>	Cape chacma baboon	2	Dada et al., 2018
Cheirogaleidae	<i>Cheirogaleus medius</i>	Fat-tailed dwarf lemur	*	Kimura, 1994
	<i>Microcebus murinus</i>	Gray mouse lemur	13*	Kimura, 1994; Neufuss et al., 2014; Petter and Jouffroy, 1993
Galagidae	<i>Galago senegalensis</i>	Senegal bushbaby	23	Ariano et al., 1973; Edgerton et al., 1975a; Hemingway et al., 2020; Huq, 2013; Huq et al., 2018; Kimura, 1994; Sickles and Pinkstaff, 1981
	<i>Otolemur crassicaudatus</i>	Brown greater galago	2*	Hemingway et al., 2020; Kimura, 1994; Muchlinski et al., 2018

		<i>Otolemur garnettii</i>	Northern greater galago	*	Kimura, 1994
	Hominidae	<i>Gorilla gorilla</i>	Western gorilla	5	Omstead et al., 2019; Potau et al., 2011; this study
		<i>Homo sapiens</i>	Human	309*	Arbanas et al., 2010, 2009; Bloemberg and Quadrilatero, 2012; Boyd-Clark et al., 2001; de Diego et al., 2020; Edgerton et al., 1975b; Edström and Nyström, 1969; Gómez et al., 2022; Hesse et al., 2013; Johnson et al., 1973; Jørgensen et al., 1993; Kimura, 2002; Kohn et al., 2011a; Levine et al., 2003; Lexell et al., 1983; Plas et al., 2015; Potau et al., 2018; Rantanen et al., 1994; Rowleron et al., 1983; Širca and Kostevc, 1985; Srinivasan et al., 2007; Thorstensson and Carlson, 1987; Tirrell et al., 2012; Zheng et al., 1992
		<i>Pan troglodytes</i>	Chimpanzee	29*	de Diego et al., 2020; Gómez et al., 2022; Myatt et al., 2011; Neufuss et al., 2014; Omstead et al., 2019; O'Neill et al., 2017; Potau et al., 2018; Rowleron et al., 1983
		<i>Pan paniscus</i>	Bonobo	1	Neufuss et al., 2014
		<i>Pongo abelii</i>	Sumatran orangutan	2	Myatt et al., 2011; Neufuss et al., 2014
		<i>Pongo pygmaeus</i>	Bornean orangutan	4	Kimura, 2002; Potau et al., 2011
			Orangutan	*	Omstead et al., 2019
	Hylobatidae	<i>Hylobates lars</i>	Lar gibbon	1*	Neufuss et al., 2014; Omstead et al., 2019
		<i>Nomascus gabriellae</i>	Yellow-cheeked gibbon	1	Potau et al., 2011
	Lemuridae	<i>Eulemur fulvus</i>	Common brown lemur	4	Anapol and Jungers, 1986; Neufuss et al., 2014
		<i>Eulemur macaco</i>	Black lemur	1	Hemingway et al., 2020
		<i>Hapalemur griseus</i>	Eastern lesser bamboo lemur	4	Hemingway et al., 2020; Muchlinski et al., 2018
		<i>Lemur catta</i>	Ring-tailed lemur	3	Hemingway et al., 2020; Potau et al., 2011
		<i>Varecia rubra</i>	Red ruffed lemur	1	Hemingway et al., 2020
		<i>Varecia variegata</i>	Black-and-white ruffed lemur	2	Hemingway et al., 2020; Muchlinski et al., 2018
	Lorisidae	<i>Loris tardigradus</i>	Red slender loris	*	Kimura, 1994
		<i>Nycticebus coucang</i>	Sunda slow loris	15*	Ariano et al., 1973; Huq, 2013; Huq et al., 2018; Kimura, 1994; Kimura et al., 1987; Sickles and Pinkstaff, 1981
		<i>Perodicticus potto</i>	Potto	3	Maréchal et al., 1976
	Tarsiidae	<i>Carlito syricta</i>	Philippine tarsier	2*	Hemingway et al., 2020; Muchlinski et al., 2018; Omstead et al., 2021, 2020
Proboscidea	Elephantidae	<i>Loxodonta africana</i>	African savanna elephant	1	Goto et al., 2013a
Rodentia	Bathyergidae	<i>Bathyergus suillus</i>	Cape dune mole-rat	5	Sahd et al., 2022
		<i>Cryptomys hottentotus natalensis</i>	Natal mole-rat	6	Sahd et al., 2022

	<i>Georychus capensis</i>	Cape mole-rat	6	Sahd et al., 2022
Caviidae	<i>Cavia porcellus</i>	Guinea pig	17*	Ariano et al., 1973; Asmussen and Gaunitz, 1989; Bonington et al., 1987; Gorza, 1990; Peter et al., 1972
	<i>Galea musteloides</i>	Common yellow-toothed cavy	*	von Mering and Fischer, 1999
Cricetidae	<i>Cricetus cricetus</i>	European hamster	3	Agostini et al., 1991
	<i>Mesocricetus auratus</i>	Syrian hamster	3	Mattson et al., 2002
Ctenomyidae	<i>Ctenomys talarum</i>	Talas tuco-tuco	6	Alvarez et al., 2012
Dipodidae	<i>Allactaga elater</i>	Small five-toed jerboa	2	Jouffroy et al., 2003
	<i>Jaculus jaculus</i>	Desert jerboa	10	Alnaqeeb and Al-Baker, 1994
Echimyidae	<i>Myocastor coypus</i>	Nutria	90	Tůmová et al., 2017, 2016, 2015
Gliridae	<i>Glis glis</i>	European edible dormouse	10	Malatesta et al., 2009; Popova et al., 2021
Heteromyidae	<i>Dipodomys merriami</i>	Merriam's kangaroo rat	4	Williamson and Frederick, 1977
Muridae	<i>Mus musculus</i>	Mouse (domestic)	68*	Alnaqeeb and Al-Baker, 1994; Asmussen and Gaunitz, 1989; Augusto et al., 2004; Bloemberg and Quadrilatero, 2012; Brasseur et al., 1987; Burkholder et al., 1994; Gorza, 1990; Hämäläinen and Pette, 1993; Hesse et al., 2010; Hitomi et al., 2005
	<i>Micromys minutus</i>	Harvest mouse	2	Hesse et al., 2010
	<i>Rattus norvegicus domestica</i>	Rat (domestic)	168*	Aigner et al., 1993; Almeida-Silveira et al., 1994; Ansved, 1995; Ariano et al., 1973; Armstrong et al., 1977; Armstrong and Phelps, 1984; Asmussen and Gaunitz, 1989; Bär and Pette, 1988; Bloemberg and Quadrilatero, 2012; Brigham et al., 1990; Chopard et al., 2001; Cornachione et al., 2011; Delp and Duan, 1996; Egginton, 1990; Eng et al., 2008; Fuentes et al., 1998; Gibson and Schultz, 1982; Gillespie et al., 1987; Gorza, 1990; Green et al., 1984; Hämäläinen and Pette, 1993; Hitomi et al., 2005; Ho et al., 1983; Howells et al., 1978; Larsson and Yu, 1997; Lewis et al., 1994; Melichna et al., 1987; Nemeth and Pette, 1981; Niederle and Mayr, 1978; Ozaki et al., 2001; Pousson et al., 1991; Pullen, 1977; Soukup et al., 2002, 1979; Staron et al., 1999; Thomason et al., 1986; Ustunel and Demir, 1997; Vesely et al., 1999; Wigston and English, 1992; Zacharová et al., 1997
Sciuridae	<i>Cynomys leucurus</i>	White-tailed prairie dog	35	Cotton and Harlow, 2010; Rourke et al., 2006
	<i>Cynomys ludovicianus</i>	Black-tailed prairie dog	22	Cotton and Harlow, 2010; Rourke et al., 2006
	<i>Ictidomys tridecemlineatus</i>	Thirteen-lined ground squirrel	16	Xu et al., 2013
	<i>Marmota monax</i>	Woodchuck	4	Rupert et al., 2015
	<i>Neotamias amoenus</i>	Yellow-pine chipmunk	2	Goldstein, 1971
	<i>Otospermophilus beecheyi</i>	California ground squirrel	2	Goldstein, 1971

Scandentia	Tupaiaidae	<i>Spermophilus dauricus</i>	Daurian ground squirrel	58	Chang et al., 2018; Gao et al., 2012
		<i>Spermophilus lateralis</i>	Golden-mantled ground squirrel	84	Choi et al., 2009; Goldstein, 1971; Nowell et al., 2011; Reid et al., 1995; Rourke et al., 2004
		<i>Urocitellus beldingi</i>	Belding's ground squirrel	2	Goldstein, 1971
		<i>Urocitellus undulatus</i>	Long-tailed ground squirrel	18	Lazareva et al., 2012
		<i>Tupaia everetti</i>	Mindanao treeshrew	1	Hemingway et al., 2020
		<i>Tupaia glis</i>	Common treeshrew	8	Sickles and Pinkstaff, 1981
		<i>Tupaia belangeri</i>	Northern treeshrew	2*	Schmidt and Schilling, 2007; von Mering and Fischer, 1999

* Number of individuals was not reported for all studies, therefore N is the sum of individuals from studies that reported it.

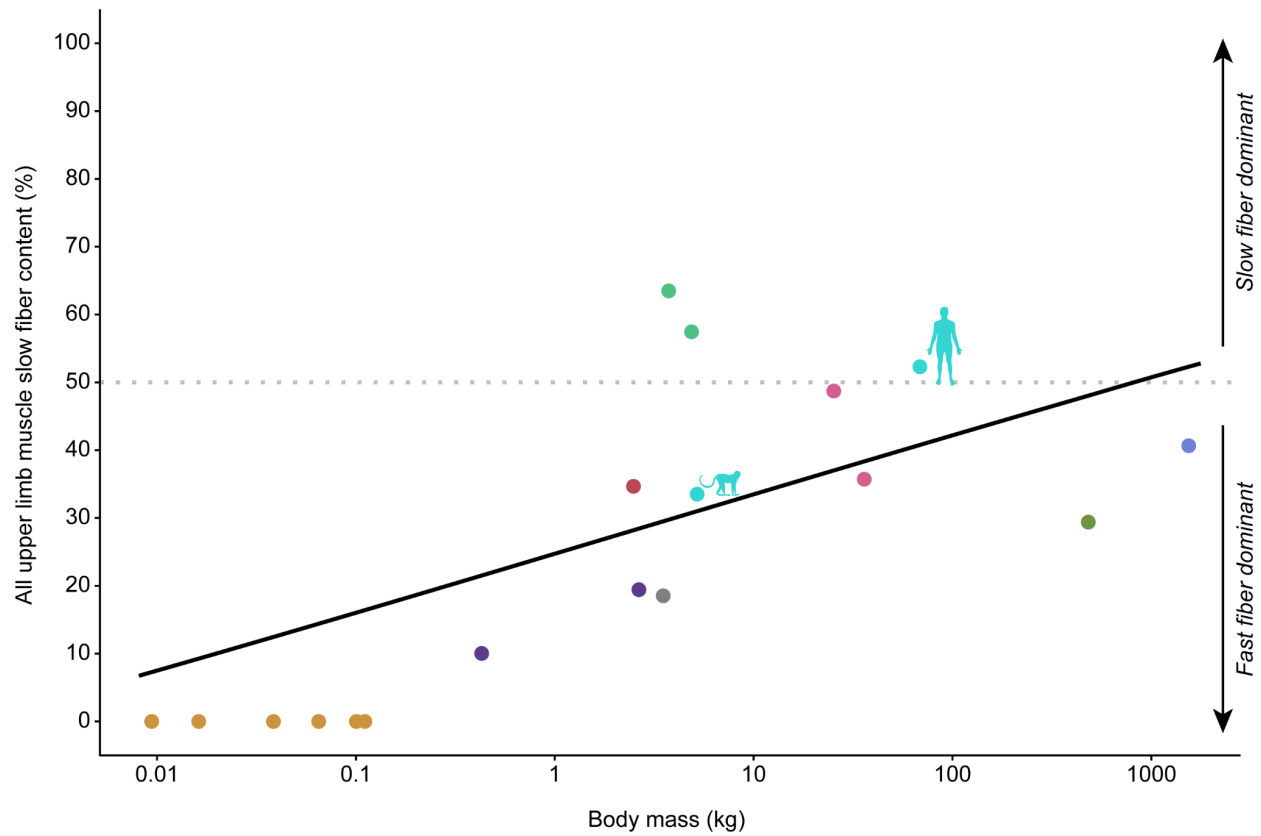
7.2 Appendix B. Studies meeting the minimum threshold ($n = 8$ upper limb muscles) for use in mammalian size scaling analysis.

Order	Common name	Scientific name	No. of subjects	Avg. body mass (kg)*	No. of muscles	Avg muscle slow myofiber content (%)	Reference
Carnivora	Dog	<i>Canis lupus familiaris</i>	3	25.3	23	48.7	Armstrong et al., 1982
	Cheetah	<i>Acinonyx jubatus</i>	2	36	15	35.7	Goto et al., 2013b
Cingulata	Nine-banded armadillo	<i>Dasyus novemcinctus</i>	4	3.51*	12	18.5	Olson et al., 2016
Didelphimorphia	Virginia opossum	<i>Didelphis virginiana</i>	7	2.488*	10	34.7	Hansen et al., 1987
Eulipotyphla	Japanese water shrew	<i>Chimarrogale platycephalus</i>	1	0.0385	10	0.0	Ichikawa et al., 2019
	Dsinezumi shrew	<i>Crocidura dsinezumi</i>	1	0.0094	10	0.0	Ichikawa et al., 2019
	Asian house shrew	<i>Suncus murinus</i>	2	0.1005	10	0.0	Ichikawa et al., 2019
	Small Japanese mole	<i>Mogera imaizumii</i>	1	0.065	10	0.0	Ichikawa et al., 2019
	Japanese mole	<i>Mogera wogura</i>	2	0.111	10	0.0	Ichikawa et al., 2019
	Japanese shrew mole	<i>Urotrichus talpoides</i>	2	0.0162	10	0.0	Ichikawa et al., 2019
	Horse	<i>Equus ferus caballus</i>	6	482	18	29.4	Kawai et al., 2009
Pilosa	Brown-throated sloth	<i>Bradypus variegatus</i>	5	3.74	11	63.5	Spainhower et al., 2018
	Hoffmann's two-toed sloth	<i>Choloepus hoffmanni</i>	4	4.875	11	57.5	Spainhower et al., 2018
Primates	Rhesus macaque	<i>Macaca mulatta</i>	3	5.2	15	33.5	Singh et al., 2002
	Human	<i>Homo sapiens</i>	6	78.5	13	47.8	Johnson et al., 1973
	Human	<i>Homo sapiens</i>	4	65.9*	11	42.7	Srinivasan et al., 2007
	Human	<i>Homo sapiens</i>	6	61.3	33	57.2	Tirrell et al., 2012
Proboscidea	African savanna elephant	<i>Loxodonta africana</i>	1	1542*	16	40.7	Goto et al., 2013a
Rodentia	Sprague Dawley rat	<i>Rattus norvegicus domestica</i>	3	0.429	16	10.0	Delp and Duan, 1996
	Woodchuck	<i>Marmota monax</i>	4	2.65*	9	19.4	Rupert et al., 2015

Order	Common name	Scientific name	No. of subjects	Avg. body mass (kg)*	No. of muscles	Avg muscle slow myofiber content (%)	Reference
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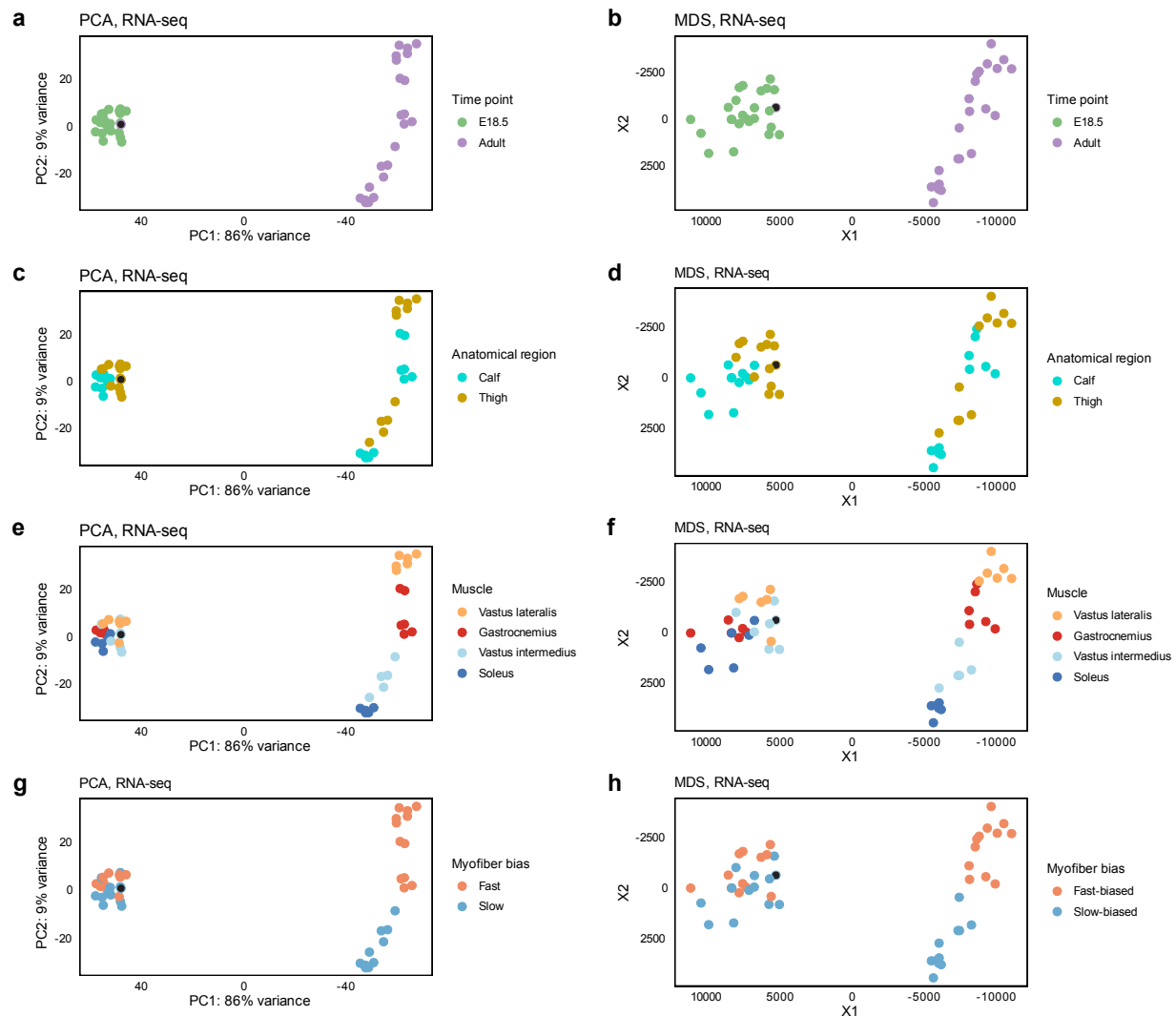
* Sample mean body mass was not provided; therefore, mass is the mean for the species.

7.3 Appendix C. Supplementary Figure 1.1.



Supplementary Figure 1.1. Size scaling of upper limb slow myofiber content across body mass ($n = 18$ species). A minimum sampling threshold of 8 upper limb muscles per species was considered representative. Dotted line represents a slow myofiber composition of 50%, above which denotes muscles that are slow myofiber dominant. The solid line represents the slope and intercept of the PGLS regression of $\log_{10}$ body mass on slow myofiber composition (estimate $\pm$ s.e. 8.61 ± 2.57 , $\lambda = 1.00$, 95% CI [0.66, NA], $r^2 = 0.38$, $t_{1,16} = 3.35$, $p = 0.004$). Data points are colored by taxonomic order (gold = Eulipotyphla; dark purple = Rodentia; gray = Cingulata; red = Didelphimorphia; bright green = Pilosa; pink = Carnivora; blue = Primates; dark green = Perissodactyla; indigo = Proboscidea). Species belonging to order Primates are distinguished with icons.

7.4 Appendix D. Supplementary Figure 4.1.



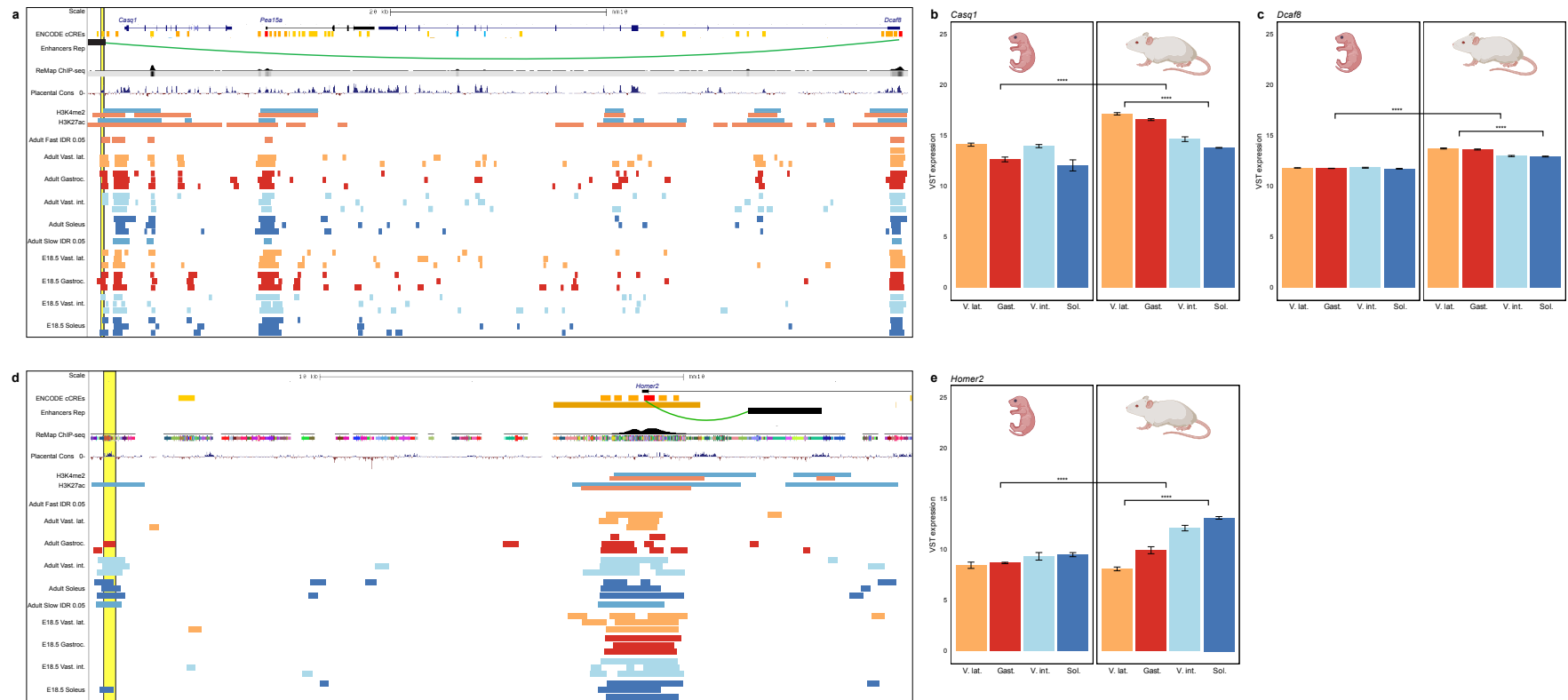
Supplementary Figure 4.1. Principal Component Analysis (PCA) and Multidimensional Scaling (MDS) of differentially expressed genes (adjusted $FDR \leq 0.05$), colored by (a,b) time point, (c,d) anatomical region, (e,f) muscle identity, and (g,h) myofiber bias ($N = 48$). The black dot represents the adult vastus intermedius sample labeled “A Vast Int 7/27/21 RNA”.

7.5 Appendix E. Supplementary Table 4.1.

Supplementary Table 4.1. Number of open chromatin peaks called per biological replicate (MACS2 $p < 0.05$) and number of biologically reproducible peaks (IDR < 0.05) before and after brain-filtering.

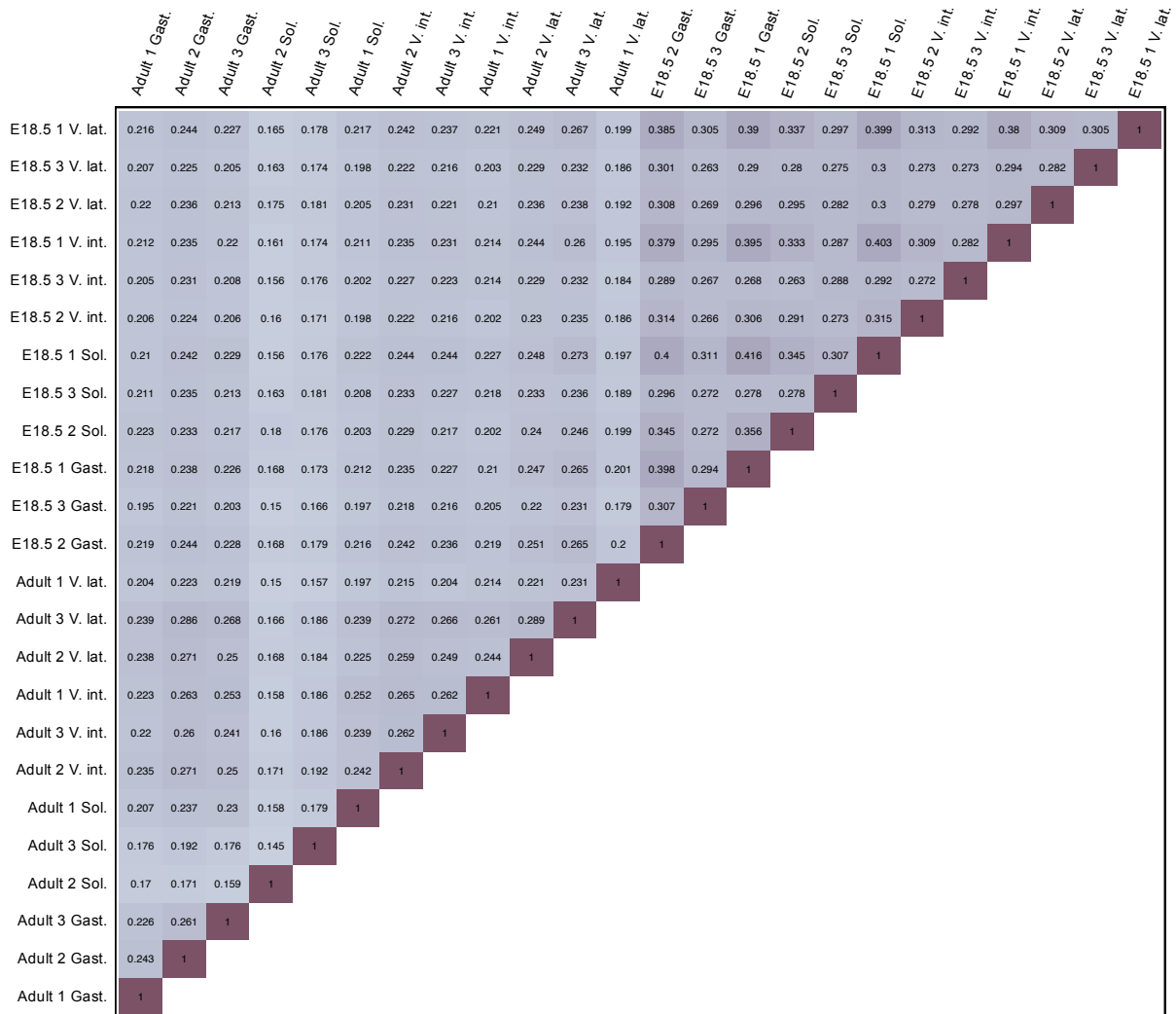
Mouse #	Muscle	Anatomical Region	Myofiber Bias	Mouse/Litter	# MACS2 Peaks ($p < 0.05$)	# IDR Peaks (IDR < 0.05)	# Brain-Filtered IDR Peaks	Average per-bp phyloP60ways conservation (Unfiltered)	Average per-bp phyloP60ways conservation (Brain-filtered IDR peaks)
E18.5	Gastrocnemius	Calf	Fast	Litter 1	211,354	49,407	33,962	0.33990	0.29586
				Litter 2	176,112				
				Litter 3	134,814				
E18.5	Vastus lateralis	Thigh	Fast	Litter 1	163,704	29,251	16,097	0.34778	0.28271
				Litter 2	149,346				
				Litter 3	178,191				
E18.5	Soleus	Calf	Slow	Litter 1	129,754	33,104	19,116	0.34679	0.28471
				Litter 2	228,137				
				Litter 3	120,387				
E18.5	Vastus intermedius	Thigh	Slow	Litter 1	175,295	32,847	19,301	0.33772	0.28070
				Litter 2	204,599				
				Litter 3	142,580				
Adult	Gastrocnemius	Calf	Fast	Adult 1	174,441	23,382	12,729	0.30645	0.23496
				Adult 2	191,754				
				Adult 3	148,259				
Adult	Vastus lateralis	Thigh	Fast	Adult 1	219,114	29,200	17,667	0.31139	0.25400
				Adult 2	187,597				
				Adult 3	155,846				
Adult	Soleus	Calf	Slow	Adult 1	149,426	15,618	6,753	0.34808	0.27474
				Adult 2	211,875				
				Adult 3	168,982				
Adult	Vastus intermedius	Thigh	Slow	Adult 1	115,333	21,926	11,351	0.32678	0.26352
				Adult 2	145,382				
				Adult 3	131,108				

7.6 Appendix F. Supplementary Figure 4.2.



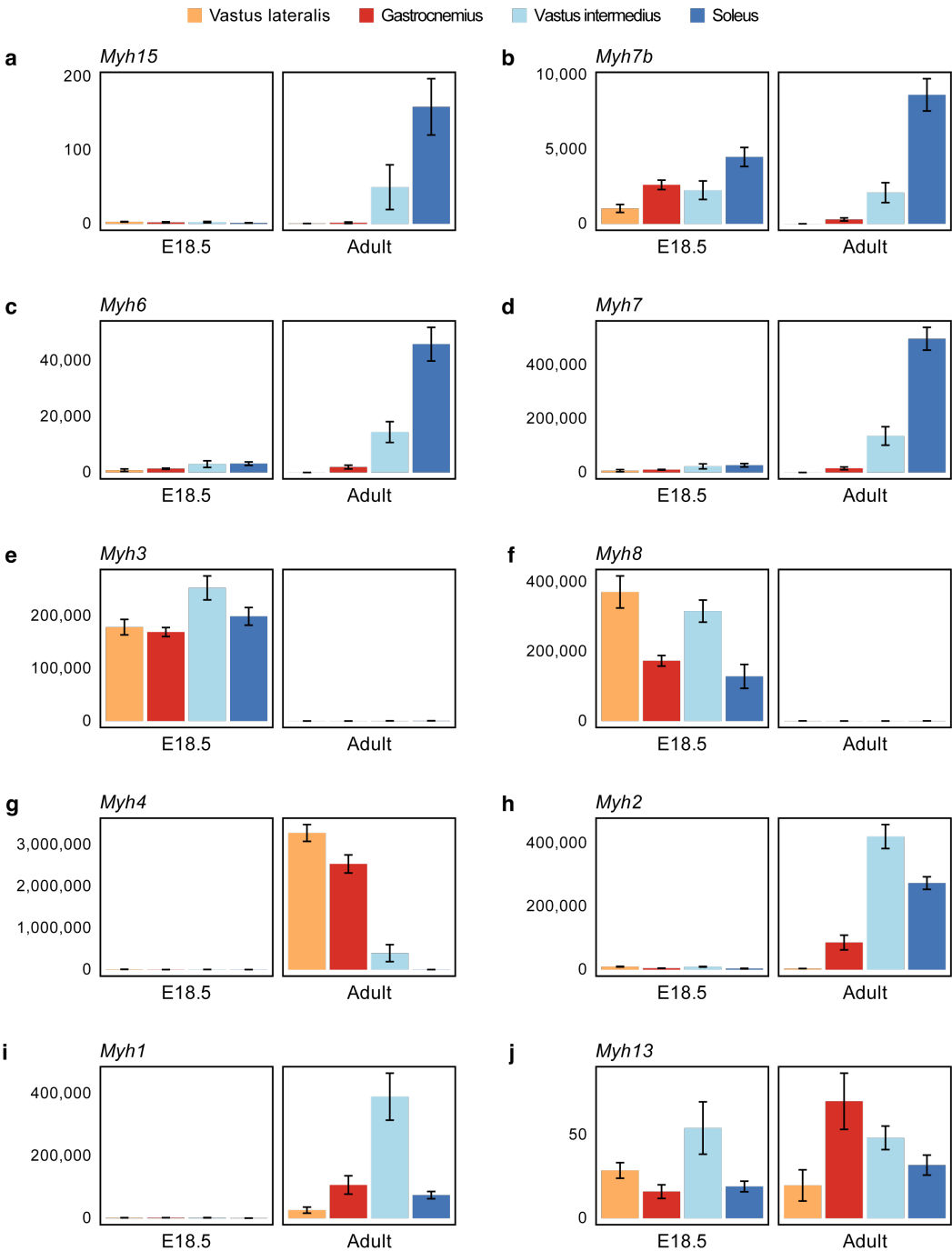
Supplementary Figure 4.2. Genomic landscape surrounding two candidate CREs upstream of DEGs. (a) ATAC-seq peak distribution, conservation scores, and publicly available mouse muscle datasets (ChIP-seq, ATAC-seq) surrounding candidate Fast 1 and (d) Slow 3. Conservation-narrowed candidate CRE regions are highlighted in yellow. Gene transcript abundance of downstream DEGs across muscle samples; (b) *Casq1*, (c) *Dcaf8*, and (e) *Homer2*. Asterisks denote statistical significance: **** $P_{adj} \leq 0.0001$. Abbreviations: V. lat., vastus lateralis; Gast., gastrocnemius; V. int., vastus intermedius; Sol., soleus.

7.7 Appendix G. Supplementary Figure 4.3.



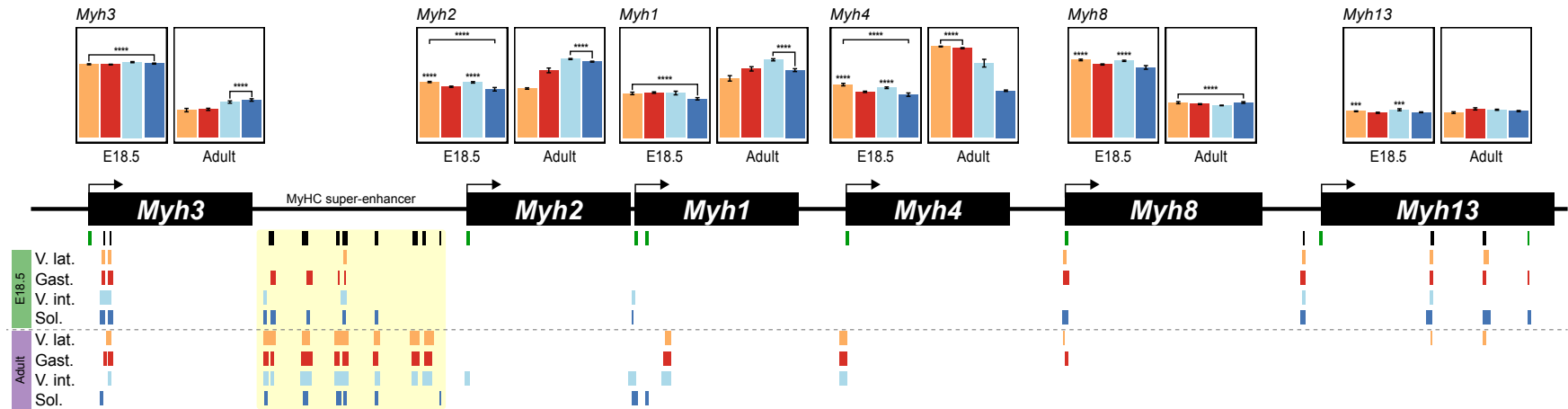
Supplementary Figure 4.3. Pairwise Jaccard index heatmap showing the overlap between MACS2 peak sets ($p < 0.05$). The color scale indicates the degree of open chromatin region overlap (light/blue = low overlap; dark/mauve = high overlap). Abbreviations: V. lat., vastus lateralis; Gast., gastrocnemius; V. int., vastus intermedius; Sol., soleus.

7.8 Appendix H. Supplementary Figure 4.4.



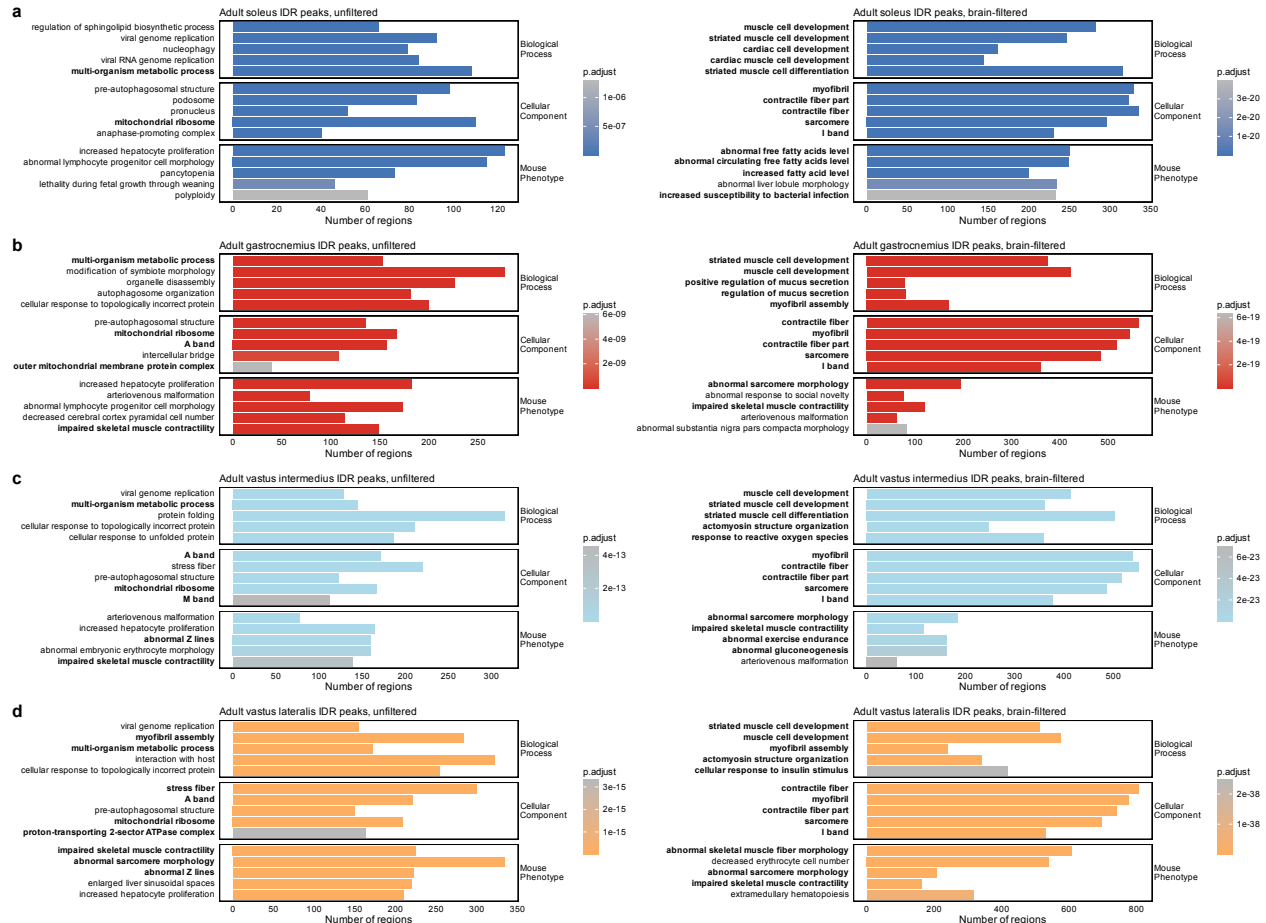
Supplementary Figure 4.4. MyHC gene counts across mouse hind limb muscles at two time points. DESeq2 library-normalized MyHC gene counts are shown for mouse hind limb muscles at E18.5 and adult time points. Vastus lateralis and gastrocnemius represent fast-biased muscles; vastus intermedius and soleus represent slow-biased muscles.

7.9 Appendix I. Supplementary Figure 4.5.



Supplementary Figure 4.5. Genomic landscape surrounding the fast 'Skeletal Muscle' MyHC gene cluster. Shown are ATAC-seq peak distributions across fast MyHC genes and the fast MyHC super-enhancer (highlighted in yellow), along with MyHC gene transcript abundance across muscle samples. Asterisks denote statistical significance: **** $P_{adj} \leq 0.0001$. Abbreviations: V. lat., vastus lateralis; Gast., gastrocnemius; V. int., vastus intermedius; Sol., soleus.

7.10 Appendix J. Supplementary Figure 4.6.



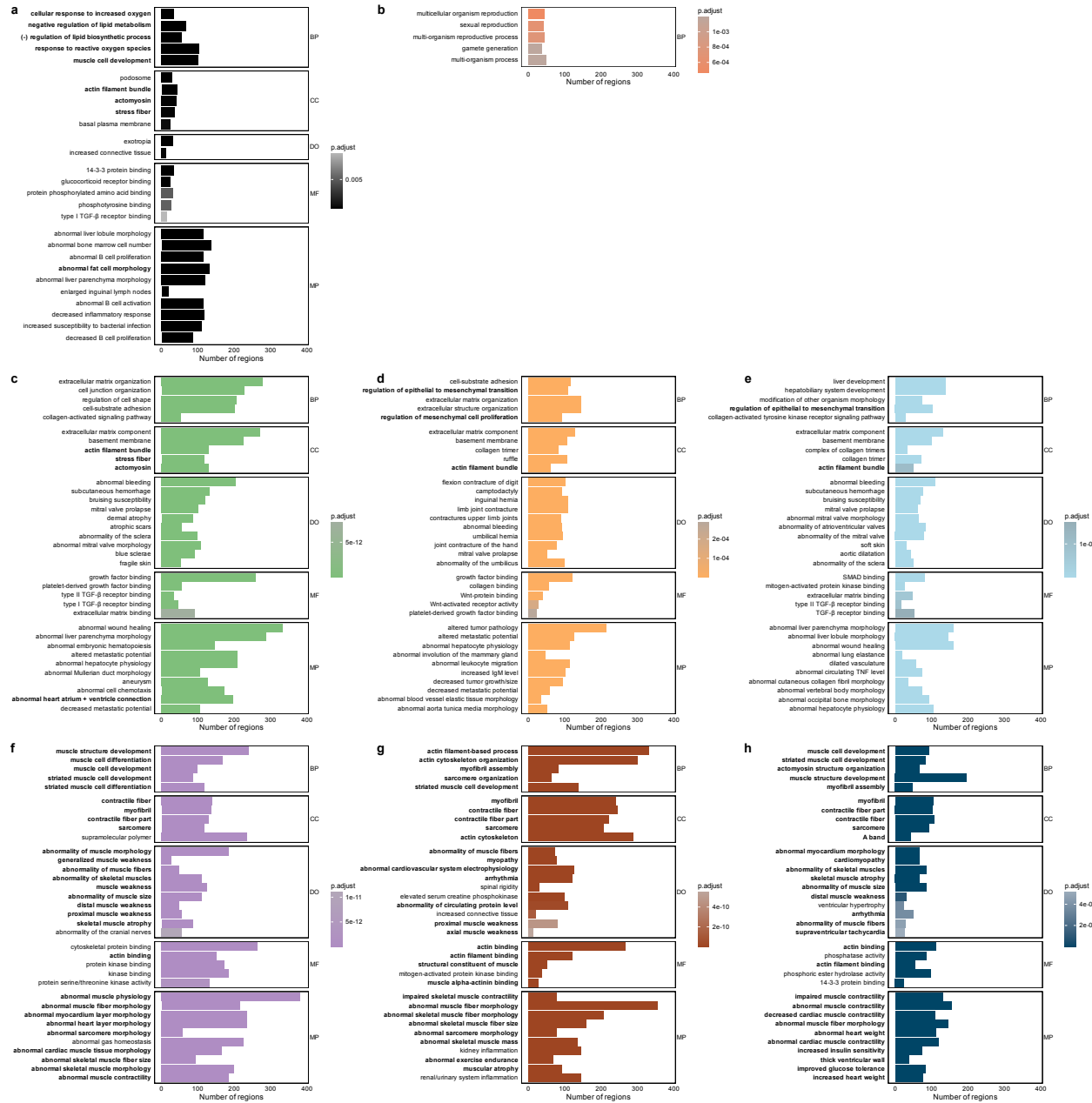
Supplementary Figure 4.6. Effect of brain-filtering on functional enrichment of IDR peak sets. Plots show the top five enriched Biological Process, Cellular Component and Mouse Phenotype terms for (a) adult soleus, (b) adult gastrocnemius, (c) adult vastus intermedius, and (d) adult vastus lateralis, before (left column) and after (right column) filtering with a mouse brain IDR peak set. Bolded terms indicate those relevant to muscle biology.

7.11 Appendix K. Supplementary Table 4.2.

Supplementary Table 4.2. Genomic feature representation (%) across brain-filtered and unfiltered IDR (FDR < 0.05) peak sets.

IDR Peak Set	Age	Muscle	Filtered/Unfiltered	Promoter	5' UTR	3' UTR	1st Exon	Other Exon	1st Intron	Other Intron	Downstream (<=300)	Distal Intergenic
Adult Gastrocnemius	Adult	Gastrocnemius	Unfiltered	48.589	0.475	1.54	2.433	2.947	7.643	12.12	0.094	24.16
Adult Soleus	Adult	Soleus	Unfiltered	57.68	0.391	0.992	2.139	2.241	6.313	10.571	0.083	19.59
Adult Vastus Intermedius	Adult	Vastus intermedius	Unfiltered	51.37	0.456	1.537	2.463	2.955	7.612	11.808	0.091	21.71
Adult Vastus Lateralis	Adult	Vastus lateralis	Unfiltered	43.36	0.455	1.966	2.428	3.586	9.342	14.517	0.127	24.22
E18.5 Gastrocnemius	E18.5	Gastrocnemius	Unfiltered	33.08	0.474	2.162	2.455	3.989	10.308	17.759	0.119	29.65
E18.5 Soleus	E18.5	Soleus	Unfiltered	43.06	0.492	1.788	2.568	3.278	8.126	13.98	0.097	26.61
E18.5 Vastus Intermedius	E18.5	Vastus intermedius	Unfiltered	42.95	0.493	1.58	2.481	3.099	8.403	13.56	0.094	27.35
E18.5 Vastus Lateralis	E18.5	Vastus lateralis	Unfiltered	46.31	0.47	1.497	2.496	2.872	7.555	12.745	0.092	25.97
Adult Gastrocnemius	Adult	Gastrocnemius	Filtered	17.92	0.526	2.333	2.899	4.784	12.609	20.049	0.141	38.738
Adult Soleus	Adult	Soleus	Filtered	18.259	0.415	1.821	2.888	4.428	12.854	21.516	0.148	37.672
Adult Vastus Intermedius	Adult	Vastus intermedius	Filtered	19.496	0.502	2.44	3.11	4.986	13.224	20.421	0.132	35.688
Adult Vastus Lateralis	Adult	Vastus lateralis	Filtered	17.326	0.492	2.728	2.796	5.338	14.162	21.922	0.164	35.071
E18.5 Gastrocnemius	E18.5	Gastrocnemius	Filtered	14.931	0.456	2.629	2.518	5.144	13.477	23.149	0.147	37.548
E18.5 Soleus	E18.5	Soleus	Filtered	17.718	0.492	2.396	2.851	4.807	12.142	20.925	0.126	38.544
E18.5 Vastus Intermedius	E18.5	Vastus intermedius	Filtered	17.574	0.492	2.078	2.736	4.528	12.492	20.087	0.119	39.894
E18.5 Vastus Lateralis	E18.5	Vastus lateralis	Filtered	18.314	0.447	2.05	2.845	4.336	11.847	20.109	0.118	39.933

7.12 Appendix L. Supplementary Figure 4.7.



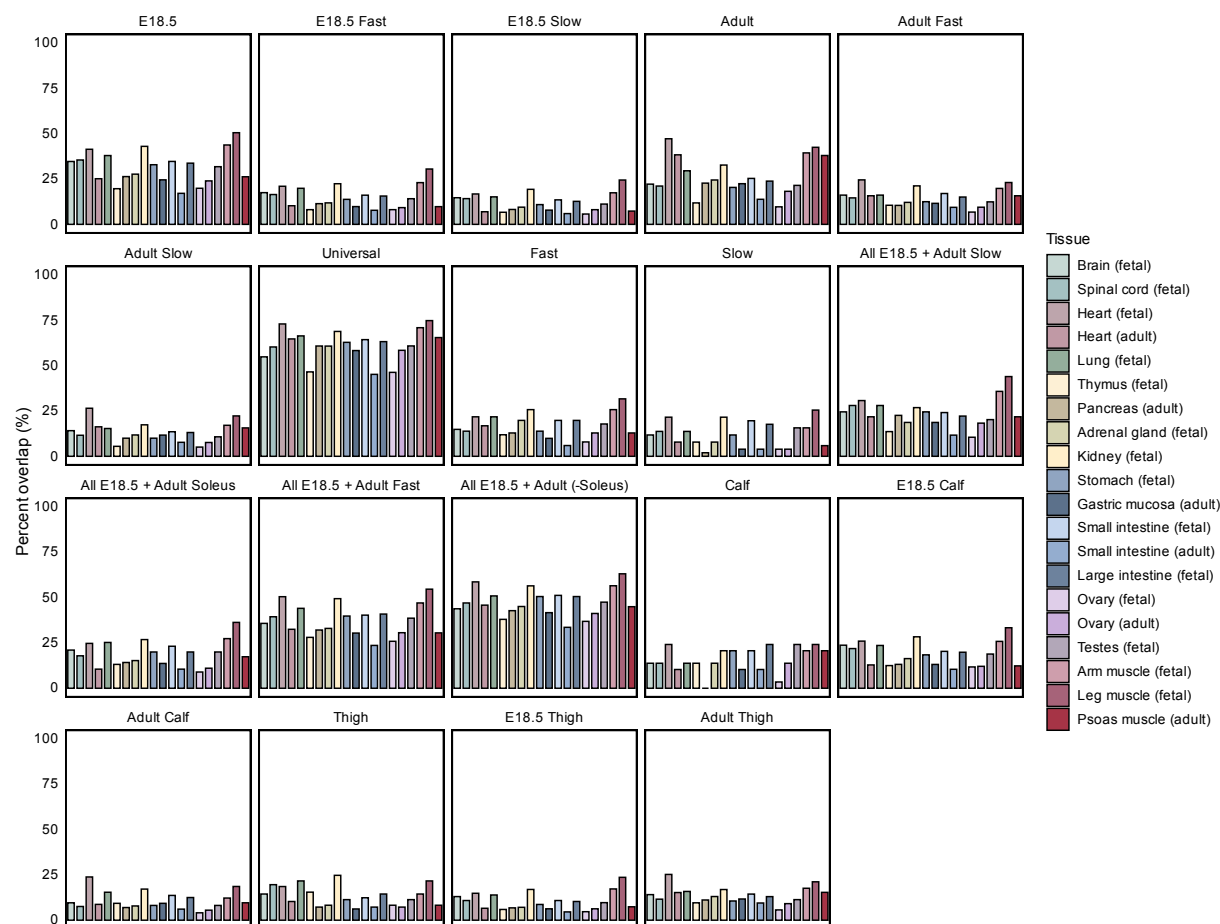
Supplementary Figure 4.7. Functional annotation of genes near the nine condition-specific peak sets. GREAT analysis showing the top enriched terms for Biological Process (BP), Cellular Component (CC), Disease Ontology (DO), Molecular Function (MF), and Mouse Phenotype (MP) for genes located near (a) Universal, (b) Fast, (c) E18.5, (d) E18.5 Fast, (e) E18.5 Slow, (f) Adult, (g) Adult Fast and (h) Adult Slow condition-specific peak sets. Bolded terms indicate those relevant to muscle biology.

7.13 Appendix M. Supplementary Table 4.3.

Supplementary Table 4.3. Genomic feature representation (%) across condition-specific peak sets.

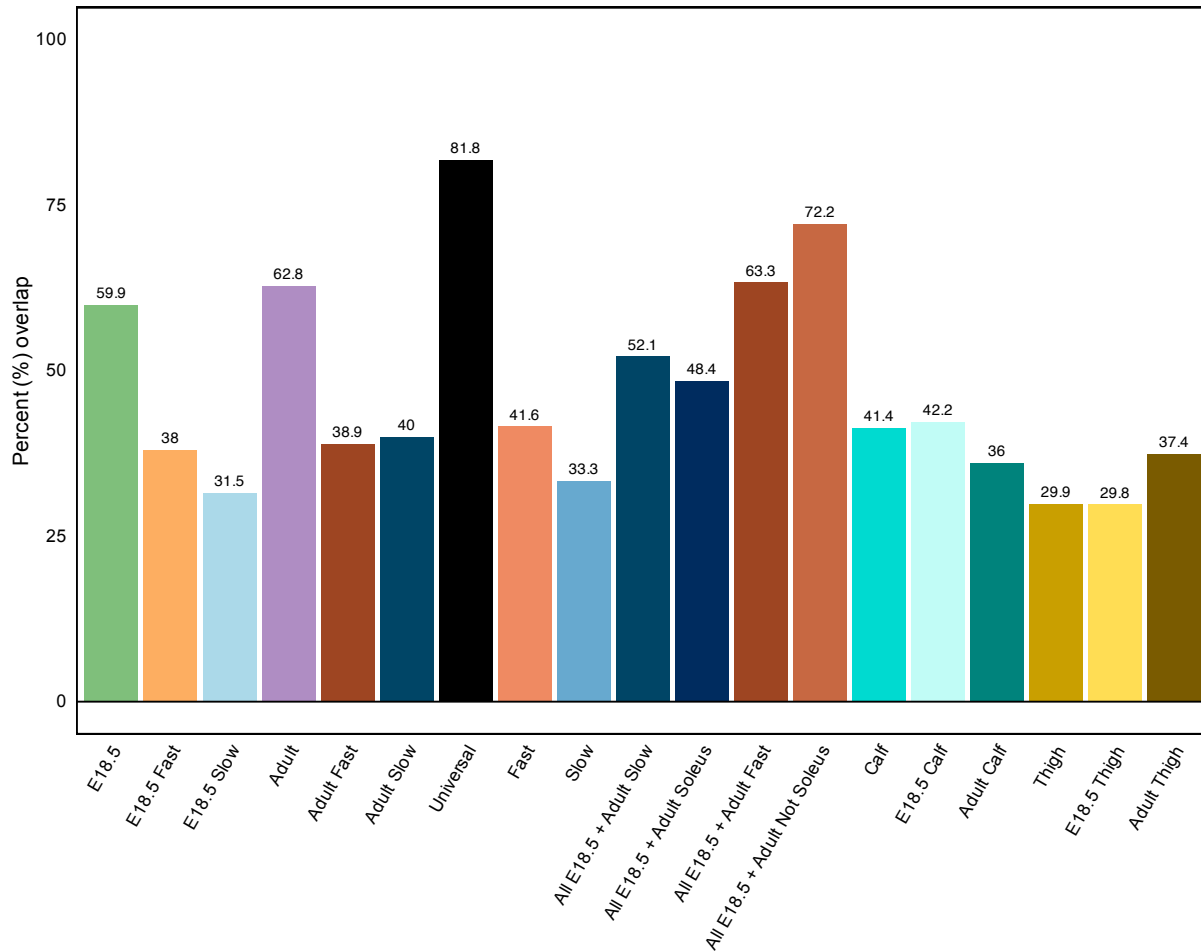
Peak Set	Time Point Specificity	Myofiber Bias Specificity	Promoter	5' UTR	3' UTR	1st Exon	Other Exon	1st Intron	Other Intron	Downstream (<=300)	Distal Intergenic
Adult Fast	Adult	Fast	14.34	0.22	2.47	1.70	4.45	18.24	27.93	0.08	30.57
Adult	Adult	Fast and Slow	16.48	0.06	2.10	2.21	3.61	17.59	26.56	0.06	31.33
Adult Slow	Adult	Slow	11.50	0.27	1.50	1.57	2.93	17.08	28.91	0.07	36.19
E18.5 Fast	E18.5	Fast	13.55	0.03	1.58	1.68	2.90	15.26	24.97	0.23	39.80
E18.5	E18.5	Fast and Slow	18.67	0.23	2.29	2.08	3.82	14.24	22.50	0.19	36.00
E18.5 Slow	E18.5	Slow	13.72	0.03	1.66	1.16	2.31	14.17	26.15	0.16	40.66
Fast	E18.5 and Adult	Fast	13.42	0.00	0.43	2.17	0.43	11.26	19.05	0.00	53.25
Universal	E18.5 and Adult	Fast and Slow	25.59	0.23	1.10	3.30	3.63	9.62	16.74	0.13	39.67
Slow	E18.5 and Adult	Slow	19.23	0.00	1.28	1.28	3.85	15.39	16.67	0.00	42.31

7.14 Appendix N. Supplementary Figure 5.1.



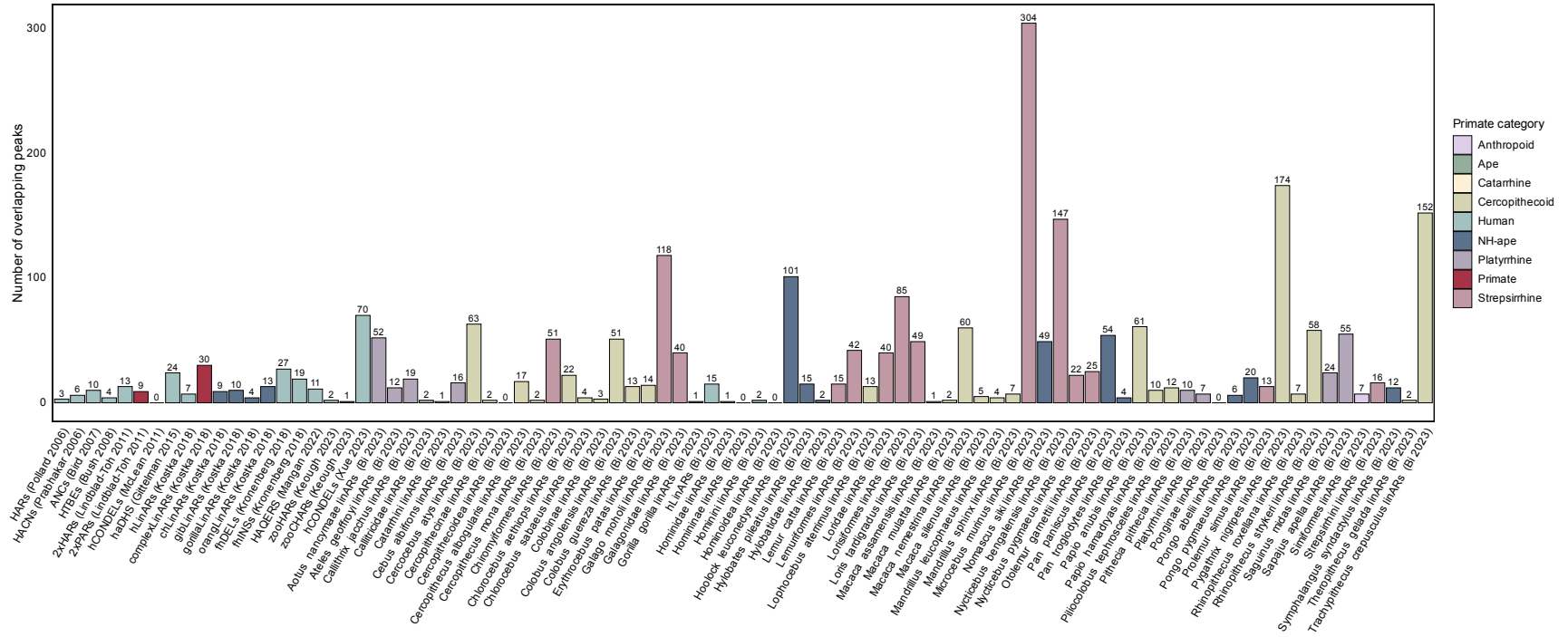
Supplementary Figure 5.1. Overlap of condition-specific peak sets with unfiltered human DNase I hypersensitivity peak sets. Proportion (%) of each condition-specific peak set overlapping merged MACS3 ($q < 0.01$) NIH Roadmap human tissue DNase I hypersensitivity peak sets prior to filtering for tissue specificity. Colors indicate distinct tissue peak sets, separated by time point (fetal, post day 100; or adult). Each panel corresponds to a different condition-specific peak set.

7.15 Appendix O. Supplementary Figure 5.2.



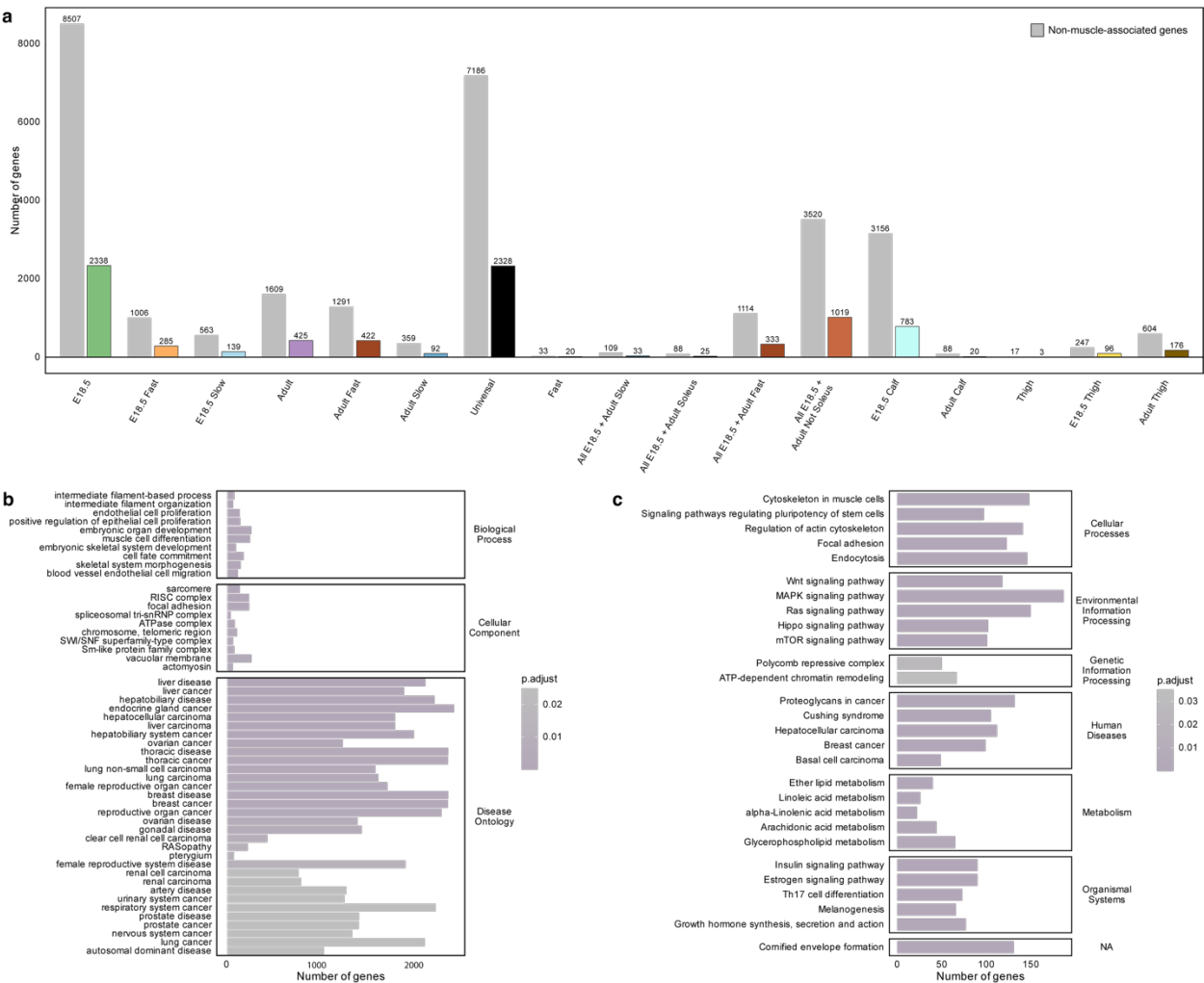
Supplementary Figure 5.2. Overlap of condition-specific peak sets with a consensus striated muscle peak set. Proportion (%) of condition-specific peak sets overlapping a merged striated muscle peak set generated from fetal heart, fetal leg muscle, fetal arm muscle, and adult psoas muscle MACS3 ($q < 0.01$) peak sets.

7.16 Appendix P. Supplementary Figure 5.3.



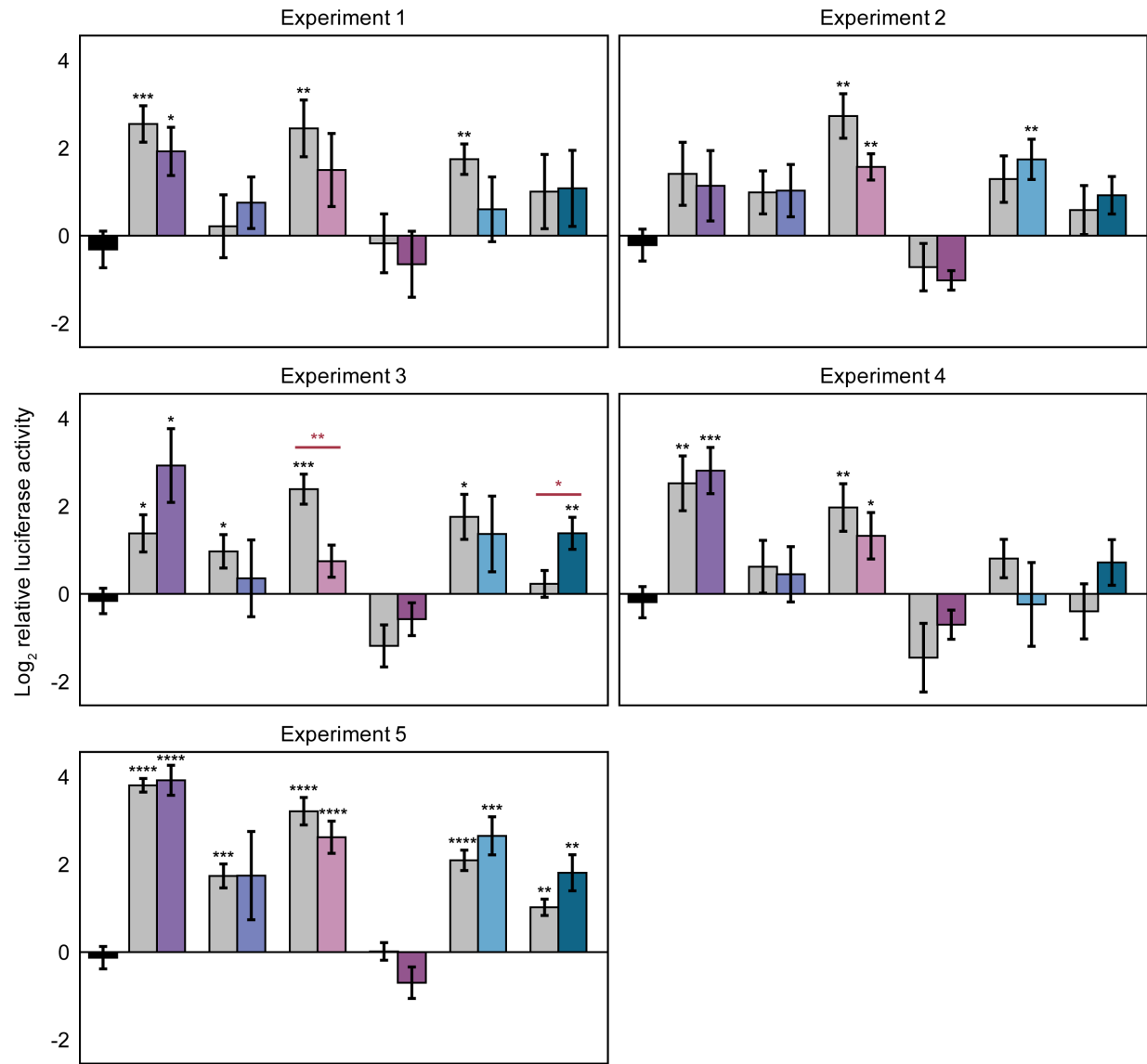
Supplementary Figure 5.3. Overlap of human striated muscle-associated condition-specific peaks with primate evolutionary datasets. Bars indicate the number of overlapping peaks for each lineage-specific dataset. Human lineage-specific datasets (e.g., HARS, hCONDELs) are shown in green.

7.17 Appendix Q. Supplementary Figure 5.4.



Supplementary Figure 5.4. Functional evaluation of genes surrounding conserved, striated-muscle accessible peaks overlapping primate signals of positive selection. (a) Total number of genes within $\pm 500,000$ bp of each primate evolutionary dataset-overlapping peak set. Colored bars indicate genes associated with muscle-related terms, while gray bars indicate genes without such associations. (b) Gene Ontology (GO) enrichment analysis of Biological Process, Cellular Component, and Human Disease Ontology terms for genes within $\pm 500,000$ bp of each peak set. (c) Top five significantly enriched pathways per KEGG category ('NA' indicates pathways not assigned to a specific category) for genes located within $\pm 500,000$ bp of each peak set.

7.18 Appendix R. Supplementary Figure 5.5.



Supplementary Figure 5.5. Functional validation of human and chimpanzee candidate CRE sequences in HSMC cells across five transfection experiments. Relative luciferase activity is shown for human (colored bars) and chimpanzee (gray bars) versions of six candidate CREs in human muscle cells (HSMC). Positive log₂ fold change (log₂FC) values indicate enhanced luciferase activity, while negative values indicate suppressed luciferase activity relative to the empty pGL4.23 control vector. Data are presented as means ± SEM (n = 6). Black asterisks denote statistically significant differences from the control vector, and red asterisks denote significant differences between human and chimpanzee sequences: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

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