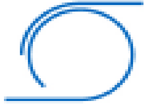

Technische Universität München
Fakultät für Sport- und Gesundheitswissenschaften

Genetics of Skeletal Muscle Phenotypes with a Focus on Hippo Genes

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// Abstract

We use our ≈650 skeletal muscles to move, stand, speak, generate force, heat, store glucose as glycogen, and amino acids as protein. Many of these functions vary greatly in the human population and twin and family studies suggest that much of this variability can be explained by genetics. Whilst transgenic mouse studies and human genome-wide association studies (GWAS) have identified some causal genes and DNA variants, much still needs to be discovered. The aim of this thesis was therefore to identify genes and DNA variants that are associated with sports and exercise-related skeletal muscle phenotypes such as muscle hypertrophy, endurance, and fiber type distribution. A special focus is on genes that are part of the so-called Hippo signal transduction network, a regulatory system involved in proliferation, stem cell function, regeneration, growth, organ size, and other phenotypes.

In the first study systematic review, I conducted a systematic review of genes whose gain or loss-of-function increases endurance capacity in mice. We found 31 genes whose manipulation increases running or swimming endurance performance by up to 1800%. Several of endurance capacity regulating genes are also associated with endurance capacity and/or VO_2max trainability indicating evolutionary conservation between humans and mice.

The second study is an experimental study in which I phenotyped and compared the muscles of hind limbs of mice where Hippo genes were mutated (*Lats1*$^{-/-}$, *Mst2*$^{-/-}$, *Vgll3*$^{-/-}$, and *Vgll4*$^{+/-}$ mutated mice) with those of wild-type control mice. I found that *Lats1*$^{-/-}$ mice had 11% more slow type I fibers and 11% fewer type IIa fibers, suggesting a slow-twitch phenotype.

Taken together, the results of this thesis give a deeper insight into the genetics of endurance performance and identify *Lats1* as a gene that contributes to determining whether a muscle is slow versus a fast-twitch muscle.

Zusammenfassung

Wir benutzen unsere 650 Skelettmuskeln, um uns zu bewegen, zu stehen, zu sprechen, Kraft und Wärme zu erzeugen, und um Glukose als Glykogen und Aminosäuren als Protein zu speichern. Viele dieser Funktionen variieren stark in der menschlichen Bevölkerung und Zwillings- und Familienstudien legen nahe, dass ein Großteil dieser Variabilität durch genetische Variabilität erklärt werden kann. Während transgene Mausstudien und humane genomweite Assoziationsstudien (GWAS) einige kausale Gene und DNA-Varianten identifiziert haben, muss noch viel entdeckt werden. Das Ziel dieser Arbeit war es daher, Gene und DNA-Varianten zu identifizieren, die mit sport- und trainingsbezogenen Skelettmuskelphänotypen wie Muskelhypertrophie, Ausdauer und Fasertypverteilung assoziiert sind. Ein besonderer Fokus liegt dabei auf Genen, die Teil des sogenannten Hippo-Signaltransduktionsnetzwerks sind, einem regulatorischen System, das an der Regulation von Proliferation, Stammzellfunktion, Regeneration, Wachstum, Organgröße und anderen Phänotypen beteiligt ist.

In der ersten systematischen Übersichtsarbeit haben wir nach Genen gesuch, deren Funktionsgewinn oder -verlust die Ausdauerleistung bei Mäusen erhöht. Wir fanden 31 Gene, deren Manipulation die Ausdauerleistung beim Laufen oder Schwimmen um bis zu 1800% erhöht. Mehrere der die Ausdauerleistung regulierenden Gene sind auch mit der Ausdauerleistung und/oder der VO2max-Trainierbarkeit assoziiert, was auf eine evolutionäre Konservierung zwischen Mäusen und Menschen hindeutet.

Die zweite Studie war eine experimentelle Studie, in der ich die Muskeln der Hintergliedmaßen von Mäusen, in denen Hippo-Gene mutiert waren (*Lats1*$^{-/-}$, *Mst2*$^{-/-}$, *Vgll3*$^{-/-}$ und *Vgll4*$^{+/-}$ mutierte Mäuse), phänotypisiert und mit denen von Wildtyp-Kontrollmäusen verglichen habe. Ich fand heraus, dass *Lats1*$^{-/-}$ Mäuse 11 % mehr langsame Typ-I-Fasern und 11 % weniger Typ-IIa-Fasern hatten, was auf einen Slow-Twitch-Phänotyp hindeutet.

Zusammengenommen geben die Ergebnisse dieser Arbeit einen tieferen Einblick in die Genetik die Ausdauerleistung und identifizieren *Lats1* als ein Gen, das dazu beiträgt zu bestimmen, ob ein Muskel ein langsam- oder schnell zuckender Muskel ist.

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Abbreviations

4E-BP1	eukaryotic initiation factor (eIF4E)-binding protein 1
ActRIIα	Activin type II receptors α
ActRIIβ	Activin type II receptors β
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATP	adenosine triphosphate
BMI	body mass index
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
$CoCl_2$	cobalt dichloride
CSA	cross-sectional area
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified Eagle's medium
DNA	Deoxyribonucleic acid
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
ERK	extracellular-regulated kinase
FBS	Fetal calf serum
FoxO	forkhead box-containing protein, O-subclass
g	grams
GAS	Gastrocnemius
GPCRs	G-protein-coupled-receptors
GSK-3	glycogen synthase kinase 3
H&E	Hematoxylin and eosin
HIF-1	hypoxia-inducible factor-1
HR	heart rate
HS	Horse serum
IFN-γ	Interferon-gamma
IGF	insulin-like growth factor
IgG	immunoglobulin G
IKK-β	IKβ Kinaseβ
IL	Interleukin
Jak	Janus kinase
KDa	Kilodaltons

Lats1/2	large tumor suppressor kinases 1 and 2
LPA	lysophosphatidic acid
MAFbx/Atrogin	muscle atrophy F-box
MAPK	mitogen-activated protein kinase
MCAT	Muscle C, A, and T sites
MEF	mouse embryonic fibroblast
MEF2	myocyte-specific enhancer factor 2
miRNA	Micro-RNA
Mob1	Msp-one-binder
mRNA	Messenger ribonucleic acid
Mst1/2	macrophage stimulating proteins 1 and 2
mTOR	mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MuRF1	Muscle RING finger 1
MW	Molecular weight
Myf 5	myogenic factor-5
MyoD	myogenic differentiation
NADH	Nicotinamide adenine dinucleotide
NFAT	nuclear factor of activated T-cells
NF2	neurofibromatosis 2
NF-κB	nuclear factor-kappa B
p70S6K1	Ribosomal S6 kinase
Pax7	paired-box 7
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PCR	polymerase chain reaction
PGC-1α	peroxisome proliferator-activated receptor-gamma coactivator 1 alpha
PI3K	phosphatidylinositol 3-kinase
PKB or Akt	protein kinase B
PPAR-β	peroxisome proliferator-activated receptor beta
PVDF	polyvinylidene fluoride
ROS	reactive oxygen species
RIPA	Radioimmunoprecipitation Assay Buffer
RNA	Ribonucleic acid
Ser	Serine
SERCA	sarco-endoplasmic reticulum calcium-ATPase

STAT	signal transducers and activators of transcription
Taz	transcriptional co-activator with PDZ binding motif
Teads	Transcriptional enhancer factor TEA domain family
TGF-β	transforming growth factor-β
Thr	Threonine
Tnnt1	troponin T1
Vgll	Vestigial-Like Family
VHL	von Hippel-Lindau tumor suppressor gene
Yap	Yes-Associated Protein

Chapter 1 General introduction

1.1 Overview

In this introduction, I will first introduce genetics and skeletal muscle as the thesis is about the effect of genetics on skeletal muscle. After that, I will more specifically discuss endurance as well as muscle mass as skeletal muscle-related phenotypes that are influenced by genetics. Next, I will specifically review Hippo genes and skeletal muscle before ending the introduction with the aims of the study.

1.2 Genetics

Humans greatly vary in their size, cold/heat tolerance, body weight, disease risks, trainability, and sporting abilities. This variation depends both on genetics, which is dependent on DNA-sequence variability within the human genome, and on environmental factors such as exercise, diet, smoking, and stress (Cowan, 1977).

Genetics is a discipline of biology that is concerned with the science of heredity including the study of genes, genetic variation, and traits of living organisms (Hartl et al., 2005, Griffiths et al., 2000). As a consequence of the sequencing of the human genome in 2000, many researchers sought to use this resource to study the impact of genetics on a variety of physiological and pathological traits such as promoting health and preventing diseases (Lightfoot et al., 2019). The study of genetic inheritance in skeletal muscle variability is no exception, with many in the science questioning the role that genetics played in skeletal muscle structure and function. The considerable genetic contribution to skeletal muscle traits suggests the possibility of using the genetic approaches to individualize therapy for myopathies or even help muscle functional limitations (Roth, 2012, Bray et al., 2009, Duan D and Mendell J R, 2019). While this possibility provides the reason and motivation to study genetics in skeletal muscle traits, to date few genes have been identified that seem to impact both muscle strength and mass (Tiainen et al., 2004, Beunen and Thomis, 2004, Roth, 2012, Duan D and Mendell J R, 2019).

1.2.1 Heritability (twin & family) studies

Twin and family studies have been used to estimate the heritability of human traits (Polderman et al., 2015, Visscher et al., 2008). Genetically, in humans, two types of twins exist which are monozygotic (i.e. genetically identical) and dizygotic (non-identical) twins. The twin studies represent comparisons between monozygotic or identical twins, who have the same genotypes/genomic sequence; and dizygotic or non-identical twins, who share almost 50% of their genotype/genomic sequences (Lightfoot J .T et al., 2019, Ye et al., 2013). It is supposed that as twins are likely to share a similar environment, the contribution of environmental factors to variability in the given phenotypes will be decreased. Thus, for a phenotype under potent genetic control, it is anticipated that monozygotic twins would represent a higher intra-pair correlation coefficient than dizygotic twins (Beunen and Thomis, 2004).

On the other hand, family studies indicate comparisons between a more expanded combination of family members than twin studies and are hence more representative of the population (Bouchard. C, 1997). Moreover, the statistical analyses allow for the identification of favorite genomic regions/sequences in relation to the phenotypes, making family studies a desirable approach. To date, human heritability estimates have been assessed for a variety of somatic treats and performance-related phenotypes, and lots of these estimates likely led to the discovery of new relations between the phenotypes and genetic polymorphisms (Silventoinen et al., 2008a, Silventoinen et al., 2003b).

Many skeletal muscle phenotypes are significantly inherited but the estimates vary greatly, highlighting that the study of heritability is not an exact science. For example, heritability estimates for muscle strength vary from 30% to 85% and for lean mass (which is mainly muscle) from 50 to 80% (Roth, 2012).

1.2.2 GWAS studies

Contrary to twin/family studies, molecular (i.e. DNA sequence) genetic studies allow researchers to investigate the role of genetic factors in unrelated persons. In the last decade, genome-wide association studies (GWAS) have become a powerful tool to identify common DNA sequence variants that influence pathological, physiological, and behavioral traits (Visscher et al., 2017, Ramanan et al., 2012).

GWAS is informative of a wide fraction of the genetic variation and allows to identify of associations between traits and so-called SNPs which are common variants of one single base. GWAS studies on diabetes, obesity, education, and height which have identified hundreds of genetic loci (i.e. points in the genome where certain SNP-bases associate with phenotypes) that associate with these traits. Fewer and often statistically underpowered GWAS studies have been performed to study sports and exercise-related traits and these have so far uncovered no/few plausible genes where SNPs associate with e.g. aerobic capacity or muscle mass (Rankinen et al., 2010, Sarzynski et al., 2016, Lightfoot J .T et al., 2019).

1.2.3 Transgenic mouse models

Associations between an SNP/gene and trait in GWAS studies do not prove causation. To study genetic causation, researchers must manipulate the relevant SNP or gene or DNA to find out whether such transgenic mutation affects the trait of interest. Specifically, researchers have developed methods to activate genes and the DNA sequences, which is known as a gain-of-function or knock-in. Conversely, other methods allow for knocking out a gene resulting in a loss of function. Such experiments can be carried out in living organisms such as mice or zebrafish or in cultured cells (Duan D and Mendell J R, 2019). In transgenic animal studies, mice are commonly used as animal models due to their availability, small size, relative ease of handling, and lower costs of husbandry as well as remarkable genomic similarities with humans (Chinwalla et al., 2002).

In 2007, Mario R Capecchi et. al received the Nobel Prize in physiology or medicine for discovering a method to create knockout mice by using embryonic stem cells. To date, this method leads to transgenic mice, and such genetically engineered animals have considerably helped us to understand the links between DNA sequences and phenotypes (Dijkstra and Walvoort, 2007).

Manipulations of genes used to be time-consuming, complicated, and expensive with older methods such as Cre-Lox recombination system, zinc-finger nucleases (ZFNs), and transcriptional activator-like effector nuclease (TALEN). More recently, clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease (Cas) 9 (CRISPR/Cas9) has emerged as a powerful tool to edit the genome in almost any species and this has made the manipulation of DNA much easier (Kim and Kim, 2014, Duan D and Mendell J R, 2019). For more details on the gene manipulation technique please see the introduction of hypertrophy systematic review (Verbrugge, 2018) and or read reviews on CRISPR-Cas9 genome editing (Khwatenge and Nahashon, 2021).

1.3 Skeletal muscle

The human body is composed of many compartments, of which, the fat-free mass compartment comprises mainly bones and skeletal muscles. The skeletal muscle is a compliant and elastic tissue that represents approximately 40% of the total body mass which is formed of muscle fibers organized in parallel. The muscle fibers are surrounded by Sarcolemma (cell membrane) and separated by the Endomysium (connective membrane). The individual muscle fibers are pulled together into bundles and surrounded by a connective tissue known as Perimysium forming skeletal muscle. Skeletal muscle is itself surrounded by another connective tissue called Epimysium (Gillies and Lieber, 2011, Chargé and Rudnicki, 2004, Sepulveda et al., 2015, Morgan and Partridge, 2020).

Skeletal muscle plays a critical role in health and well-being, including moving, maintaining posture, generating heat, protecting organs, and stabilizing joints (Sepulveda et al., 2015, Henderson et al., 2017). Moreover, skeletal muscle as a dynamic tissue is sensitive to many factors or stimuli such as nutrition, mechanical stress, and pathological states. Each factor or stimulus regulates muscle fibers by various molecular mechanisms (Bilodeau et al., 2016, Morgan and Partridge, 2020, Langridge et al., 2021) that will be described later in this chapter.

1.4 Structure of skeletal muscle

In this section, I will introduce skeletal muscle, an organ that varies greatly in muscle fiber size, fiber numbers, and fiber type percentages which are all influenced by genetics.

1.4.1 Sarcomere

Skeletal muscles contain bundles of muscle fibers. Each muscle fiber comprises myofibrils which represent a basic cellular contractile unit known as a sarcomere (Lieber et al., 2017, Sweeney and Hammers, 2018). Quick movement and force generation are features of striated muscle function conducted via contraction of the sarcomeres. They indicate a part of molecular machinery whose complex structure is formed of two basic protein filaments including thick and thin filaments that move parallel to an axis of muscle fiber. Visually, sarcomeres are defined as the region between two dark thin lines called "Z-disk". Each sarcomere consists of two halves of I-band (thin filaments) and a central A-band (thick filaments) (Henderson et al., 2017, Sweeney and Hammers, 2018) (Figure 1.1).

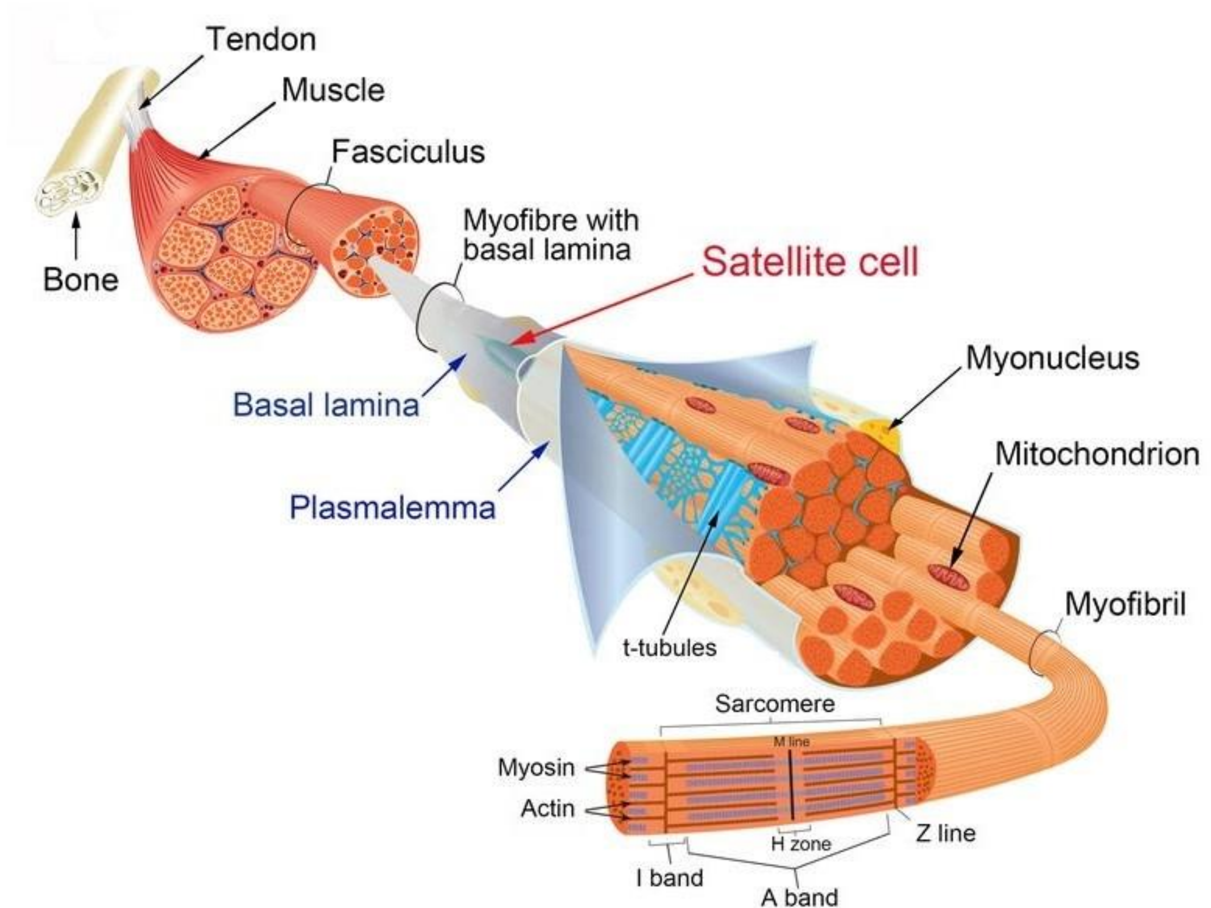

Figure 1.1. The structure of skeletal muscle.
Figure adapted from Tajbakhsh et al (Tajbakhsh, 2009).

1.4.2 Thick filament

The thick filament predominantly is composed of myosin proteins and traverses the entire length of the A band. Myosin is one of the main proteins of the M-line that is suggested to function as strain sensors within the sarcomere (Xiao and Gräter, 2014). Myosin is both functional (head) and structural (tail) protein and is associated with other non-myosin proteins such as myosin-binding proteins (MyBPs) of the M-line. MyBP-C is a main class of MyBPs that contribute to thick filament's specific organization and control force generation via the actomyosin complex (Ackermann and Kontrogianni-Konstantopoulos, 2013). MyBP-C also is associated with a flexible protein, titin. In a mature muscle, the titin extends along the length of the thick filaments and acts as a ruler, to define the precise organization and length of thick filaments (Henderson et al., 2017, Mukund and

Subramaniam, 2020). Moreover, one of the main proteins of the M-line that acts as strain sensors within the sarcomere is myomesin (Xiao and Gräter, 2014). Thick filaments are linked at the M-line with anti-parallel dimers of myomesin, and they are linked in a triplex complex with titin and obscurin (Gautel and Djinović-Carugo, 2016, Pernigo et al., 2015, Pernigo et al., 2017) (Figure 1.2).

1.4.3 Thin filament

Thin filaments are an essential part of the contraction machinery that consists of globular actin (G actin) subunits, which polymerize into strands and twist to form an α helical structure. Thin filaments are associated with a group of regulatory proteins including tropomyosin and troponin that facilitate contraction. The most important are troponin and tropomyosin. Troponin is composed of three subunits, including troponin I (TNN-I) an inhibitory subunit that binds to actin; troponin T (TNN-T), the tropomyosin binding component to position it on actin; and troponin C (TNN-C), which binds calcium ions (Ca^{2+}), and tropomyosin functions to stabilize the actin and blocks the active sites of actin to prevent attachment between the myosin cross-bridges and the thin filaments (Zot and Potter, 1987, Mukund and Subramaniam, 2020). Tropomodulin is another important thin filament protein, which regulates the precise filament length for efficient contraction (Gokhin et al., 2015). Nebulin also is an actin-binding protein that can bind up to 200 actin molecules and its length is proportional to the length of the thin filament (Labeit et al., 2011) (Figure 1.2).

1.4.4 T-tubules

Besides architectural proteins, muscle fiber sarcoplasm contains organelles essential for contraction. Among these, T-tubules (transverse tubules) are tubules organized from the same phospholipid bilayer as the surface membrane (sarcolemma) of skeletal muscle fibers (Hong and Shaw, 2017) (Figure 1.1). The T-tubule network is an extension of the sarcolemma that penetrates into the middle of the skeletal muscle cells. With these membranes that contain considerable concentrations of transporters, pumps, and ion channels, T-tubules allow the quick transmission of the action potential and play a key role in regulating the concentration of cellular calcium (Frontera and Ochala, 2015, Hong and Shaw, 2017).

1.4.5 Z-disk

The Z-discs (Z-disk, Z-line, Z-band) are dark lines under the electron microscope that delineate the lateral borders of sarcomeres of muscle tissue, and cross-link anti-parallel actin filaments in a

regular lateral array (Luther, 1991). The Z-disc has primarily been considered important for mechanical stability. Z-disk directly binds to several proteins such as F-actin filaments, myozenin, γ-actin, γ-filamin, α-actinin, myopodin, myopalladin, myotilin, (Papponen et al., 2009, Savarese et al., 2016) desmin, muscle LIM protein (MLP), nebulin, actin, titin, and nuclear receptor interaction protein (NRIP protein) which are critical for sarcomere mechanics (Chen et al., 2015, Knöll et al., 2011) (Figure 1.2).

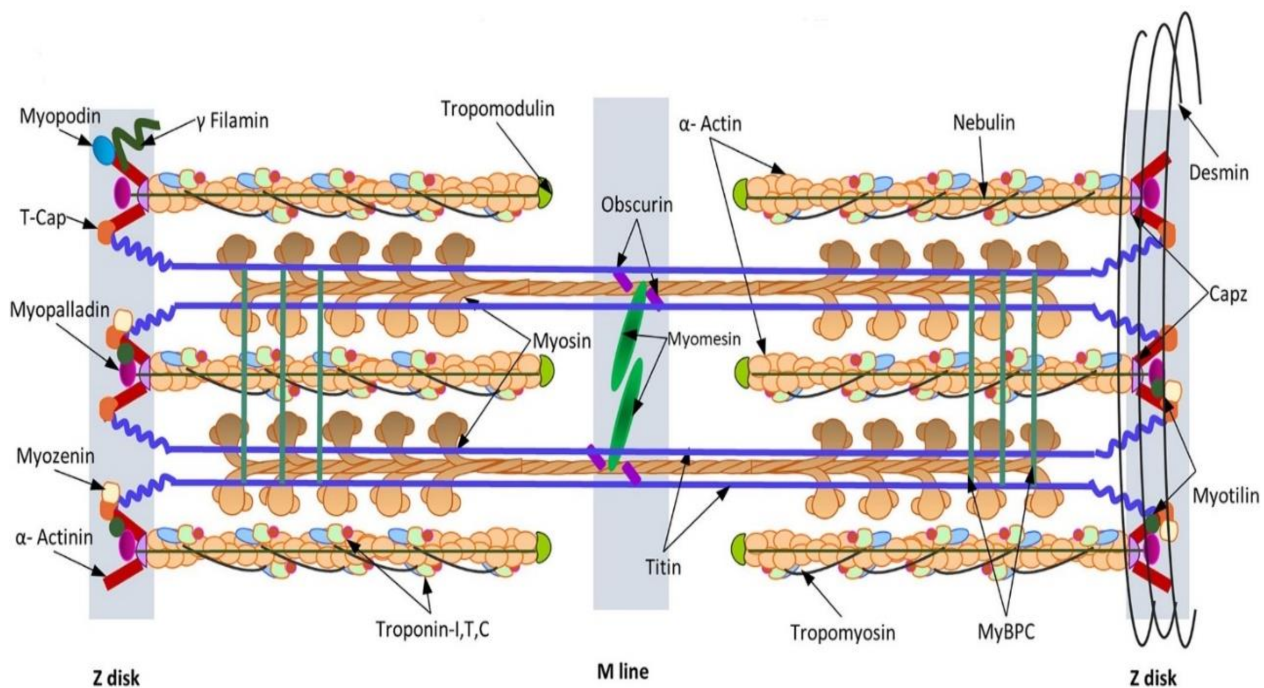

Figure 1.2. the structure of muscle sarcomere.
Schematic representation of the skeletal muscle sarcomere showing the arrangement of thick and thin filaments in the sarcomere, and identifying the location of major sarcomeric proteins. Figure adapted from Mukund et al (Mukund and Subramaniam, 2020)

1.5 Skeletal muscle fiber types

Skeletal muscle is composed of different fiber types with different features such as oxidative capacity, force generation, color, shortening velocity, and fatigability (Schiaffino and Reggiani, 2011). Based on this, major types of skeletal muscle fibers are identified: (a) Slow-twitch fibers, fatigue-resistant with high oxidative metabolism and low mechanical power output (b) fast-twitch, quickly fatigable fibers with high glycolytic (anaerobic) metabolism and high mechanical power output, and (c) fast-twitch intermediate fibers which combine resistance to fatigue and high power output (Schiaffino et al., 2013, Zierath and Hawley, 2004, Blaauw et al., 2013, Sher and Cardasis, 1976, Henderson et al., 2017).

Physiologically, the difference between slow-twitch and fast-twitch muscle fibers is based on differences in their molecular motor activity, calcium kinetics, and excitation-contraction coupling (ECC) mechanisms that direct the basic twitch parameters including half-relaxation and peak tension times. Fast-twitch muscle fibers, for instance, show a rapid contraction of the sarcomere and shorter twitch parameters through the greater abundance of the ryanodine receptors (RYRs, calcium release channels) and Sarco/endoplasmic reticulum $Ca2^{+}$-ATPase pumps (SERCAs pumps) (Reggiani and te Kronnie, 2006). These fibers are also presenting higher mechanical power and faster contractile machinery due to differing myosin heavy chain isoforms (*MYH4* in IIB, *MYH1* in IIX, and *MYH2* in IIA fibers, respectively). However, slow-twitch muscle fibers contain type I myosin heavy chain (*MYH7*), contract much more slowly, and generate less mechanical power with lesser ATP expenditure (Rivero et al., 1998, Schiaffino, 2010). Genetically, each muscle fiber type is equally diverse with different thin and thick filament isoforms which are expressed in fast and slow muscles. For example, *TPM1, CASQ1, MYL1, MYBP2, ATP2A1, TNNT3/I1/C2,* and *TMOD4* genes exhibit fast-twitch muscle fiber isoforms, while *TPM3, CASQ2, MYBL2, MYL2/3, ATP2A2, TNNT1/I1/C1,* and *TMOD1* genes exhibit slow-twitch muscle fiber isoforms (Schiaffino, 2010, Schiaffino and Reggiani, 2011, Bottinelli and Reggiani, 2000, Mukund and Subramaniam, 2020).

1.6 Skeletal muscle and endurance capacity

Endurance in skeletal muscle has been linked to type I and II subtype muscle fiber proportions (Costill et al., 1976), muscle capillary density (Brodal et al., 1977), mitochondrial and other metabolic enzyme activities (Gollnick and Saltin, 1983) as well as the glycogen concentration of the exercising muscles (Bergstrom et al., 1967).

Given that endurance capacity is a multi-factorial trait, apart from skeletal muscle, several sub-traits and organ systems need to be considered as follows:

1) **Aerobic capacity (VO_2max)** is influenced by the maximal cardiac output and by the oxygen transport capacity of the blood, and blood volume (Bergh et al., 2000, Lundby et al., 2017).
2) **Mechanical efficiency** describes how much chemical energy is converted into mechanical power (Bassett and Howley, 2000). Efficiency depends on many factors including body weight and height (Maldonado et al., 2002).
3) **Mental endurance** depends on the nervous system and is defined as fatigue resistance during prolonged periods of demanding cognitive activity (Van Cutsem et al., 2017).

In relation to human endurance, two important questions are: How much is endurance inherited? What DNA sequence variants affect endurance capacity? Classical genetic studies suggest that maximal aerobic performance variables (i.e. VO2max, physical working capacity, or threshold values) are between 38 and 94% inherited (Peeters et al., 2009). In the Heritage Study, Bouchard et al. estimated that the VO_2max was 50% inherited (Bouchard et al., 1998) and that the VO_2max trainability was 47% inherited (Bouchard et al., 1999). Similarly, the muscle fiber distribution was estimated to be ≈45% inherited (Simoneau and Bouchard, 1995). Collectively, especially the Heritage study data suggest that the variation of major human endurance-related traits depends probably up to ≈50% on DNA sequence variation (i.e. genetics (Bouchard et al., 1998, Bouchard et al., 1999, Simoneau and Bouchard, 1995) implying that ≈50% is dependent on environmental factors such as endurance training and nutrition.

We still incompletely understand the genetics of human endurance and some researchers are even skeptical about the importance of genetics. To identify genes and alleles that can have a major effect on endurance it is especially useful to review the data of transgenic mouse studies (Garton et al., 2016). In transgenic mouse studies, genes are manipulated to produce gain or loss-of-function of a gene. If this results in a measurable increase of endurance, then the gene is a candidate gene for human endurance capacity, too. In Chapter II, the available literature concerning genes whose gain or loss-of-function increases endurance capacity will be reviewed.

1.7 Regulation of muscle mass

The maintenance of skeletal muscle mass is governed by the result of a balance between protein synthesis and degradation (Murton and Greenhaff, 2013). A shift in this balance activates either anabolic or catabolic signaling pathways which lead to visible changes in appearance, performance, and composition of the muscle fiber via external and internal factors such as steroids (Yu et al., 2014), growth factors, inflammation (Haddad et al., 2005, Jackman and Kandarian, 2004, Sartori et al., 2021, Yoshida and Delafontaine, 2020), oxidative stress (Steinbacher and Eckl, 2015, Scicchitano et al., 2018), Glucocorticoids (Alev et al., 2018, Quattrocelli et al., 2017, Fappi et al., 2019) and diseases (Bailey et al., 2006, Doucet et al., 2007, Langridge et al., 2021).

1.7.1 Muscle hypertrophy signaling pathways

Skeletal muscle hypertrophy (anabolism) can be defined as an increase in the size of muscle fibers that occurs as a result of the accumulation of contractile and other cytoskeleton proteins within the fiber. The major part of the studies on muscle hypertrophy has focused on networks that regulate mRNA translation initiation. These studies have identified the mammalian target of rapamycin complex 1 (mTORC1) as an important hub integrating upstream signaling networks that result in protein synthesis. The main targets of mTORC1 that function as key regulators of translational efficiency are 4E binding protein (4E-BP1), ribosomal S6 kinase ($p70^{S6K1}$), eukaryotic initiation factor 2 (eIF2), and 3 (eIF3f) (Ma and Blenis, 2009, Sartori et al., 2021). Studies suggested that mTORC1 signaling is up-regulated in different models of skeletal muscle hypertrophy, while this signaling is down-regulated during atrophy (Yoshida and Delafontaine, 2020, Drummond et al., 2009, Trendelenburg et al., 2009).

The observations indicated that mTORC1 is necessary and sufficient to induce muscle hypertrophy in various physiological conditions. Hence, several studies showed that protein synthesis induced by the insulin-like growth factor 1 (IGF-1), Clenbuterol treatment, and mechanical loading, are markedly inhibited after rapamycin treatment as an inhibitor of mTORC1(Nader et al., 2005, Drummond et al., 2009, Hornberger et al., 2003, Kline et al., 2007, Sartori et al., 2021).

mTORC1 is governed through a variety of upstream molecules which the best characterized of them is the IGF-1. Evidence has revealed growth factors, such as IGF-1, induce muscle hypertrophy through the activation of the signaling pathway involving protein kinase B (PKB or Akt) and,

phosphoinositide 3-kinase (PI3K) which lie genetically upstream of mTORC1(Bodine et al., 2001, Rommel et al., 2001, Sartori et al., 2021, Gonçalves et al., 2019). Furthermore, mTORC1 is regulated by amino acids. Elevated levels of intercellular amino acids, especially leucine, are able to activate mTORC1 signaling in a PIK3/Akt independent manner (Dreyer et al., 2008, Kim et al., 2002). Findings have shown that Rag small GTPase, vacuolar protein sorting 34 (Vps34), and Ras homolog enriched in brain (Rheb) proteins that act as amino acid 'sensors' can activate mTORC1 signaling (Kim, 2009, Dreyer et al., 2008, Kim et al., 2002). Some studies have also indicated that wingless-related integration site (wnt) ligand 7a (Wnt7a) is able to induce hypertrophy in skeletal muscle via its receptor frizzled 7 (Fzd7), which leads to activation of PI3K/Akt/mTORC1 signaling network (von Maltzahn et al., 2011).

Mechanical loading/stretching is another potent mTORC1activator and protein synthesis. Evidence has suggested phospholipase D (PLD) and its metabolite phosphatidic acid (PA) as important molecules in loading/stretching induced hypertrophy in skeletal muscle (O'Neil et al., 2009, Hornberger et al., 2006, Hornberger et al., 2004). Activation of mTORC1 signaling in response to mechanical loading of cultured muscle was highly reduced by inhibiting PLD (Hornberger et al., 2004, Hornberger et al., 2006, O'Neil et al., 2009). Another study also has shown that PLD is necessary for mTORC1 activation via Rheb in skeletal muscle(Sun et al., 2008, Hong-Brown et al., 2013), indicating PLD seems to describe a molecular link between mTORC1 signaling and mechanical loading (Frias et al., 2020).

In addition to AKT/mTOR signaling, muscle hypertrophy is induced due to satellite cells activation through G-protein coupled receptors, particularly via the α-subunit $G\alpha_{i2}$(Minetti et al., 2014, Minetti et al., 2011) and myostatin inhibition (Amthor et al., 2009, Zhu et al., 2000). $G\alpha_{i2}$ in a PKC and HDAC4-dependent manner affects downstream signaling of mTOR resulting in hypertrophy (Minetti et al., 2014).

1.7.2 Muscle atrophy signaling pathways

Skeletal muscle atrophy occurs when the mechanisms that regulated protein degradation (catabolic pathways) prevail over those which regulated protein synthesis (anabolic pathways). Atrophying muscle is characterized by a loss of muscle mass, a decrease in the muscle fiber cross-sectional area (CSA), and the amount of muscle protein (Boonyarom and Inui, 2006, Jackman and Kandarian, 2004). The master regulators of muscle atrophy are the

activation of the ubiquitin-proteasome system (Lecker et al., 2004, Sandri et al., 2004, Sartori et al., 2021). In skeletal muscle, Atrogin-1 and muscle ring finger protein-1 (MuRF1) represent two E3 ubiquitin ligases of the ubiquitin-proteasome system that act by targeting the eukaryotic translation initiation factor 3 (eIF-3) and myosin chains respectively for protein degradation (Cohen et al., 2009, Foletta et al., 2011).

Pro-inflammatory cytokines such as interleukin1 (IL-1), IL-6, and TNFα promotes the expression of *Atrogin-1* and *MuRF1* as key muscle-specific E3 ligases via p38 mitogen-activated protein kinases (p38MAPK) (Li et al., 2005), Forkhead box-containing protein, O4 (FoxO4) (Moylan et al., 2008) and Nuclear factor-kappa β (NF-kβ) (Ma et al., 2017, Jackman et al., 2013) signaling pathways that result in muscle atrophy.

It has been shown that activation of NF-kβ, through its upstream IKβ Kinaseβ (IKKβ) induces notable muscle atrophy in the mouse model (Cai et al., 2004), while expression of a dominant negative IKKβ markedly reduces muscle atrophy in response to muscle disuse (Van Gammeren et al., 2009). The NF-kβ pathway is thought to promote atrophy by governing the expression of some ubiquitin ligases, such as *MuRF1* (Cai et al., 2004), as well as inhibiting myogenic differentiation by repressing the function of *MyoD* (Wang et al., 2007, Guttridge et al., 2000, Langen et al., 2001). These findings have suggested a pivotal role for NF-kβ in muscle atrophy.

IL-6 also induces muscle atrophy by regulating the JAK/STAT(Haddad et al., 2005) pathway. A recent study has highlighted improving denervation-induced atrophy via inhibiting IL-6-STAT3 signaling in Fibro–adipogenic progenitors (FAPs) (Marazzi and Sassoon, 2018, Madaro et al., 2018), which emphasizes the impact of immune signaling on muscle atrophy.

Another ubiquitin ligase E3, FBXO40, is suggested to trigger muscle atrophy in denervated muscle by suppressing the PI3K/AKT pathway through ubiquitinating insulin receptor substrate (IRS1)(Ye et al., 2007).

Like NF-kβ, FoxO transcription factors are highly expressed in various types of muscle atrophy. In-vivo studies suggested that over-expression of FoxO1/3 induces notable reductions in mice muscle mass and myofibre CSA (O'Neill et al., 2016, O'Neill et al., 2019, Zhao et al., 2007b). In vitro analysis also has shown that over-expression of FoxO stimulates protein degradation, whereas knock-down of FoxO1 and FoxO3 decreases proteolysis in myotubes (Zhao et al., 2007b, Smith et al., 2010, Sartori et al., 2021). *FoxO* transcription factors are able to up-regulate *Atrogin-1* and *MuRF*1 expression by directly binding to their promoter regions (Brault et al., 2010, Sandri et al.,

2004, Waddell et al., 2008, Pomiès et al., 2016). Interestingly, FoxO transcriptional activity is negatively regulated by the IGF-1/PI3K/Akt pathway through direct phosphorylation by Akt (Sandri et al., 2004, Stitt et al., 2004). Conversely, reduced AKT activation also permits phosphorylation and translocation of FOXO to the nucleus which is sufficient to promote *MuRF1* and *Atrogin-1* expression in atrophic conditions (Sandri et al., 2004, Stitt et al., 2004, Ikeda et al., 2016).

Furthermore, the oxidative stress resulting from the high metabolic activity of skeletal muscle promotes atrophy in states of muscular dystrophies (Choi et al., 2016, Terrill et al., 2013, Scicchitano et al., 2018), muscle disuse (Powers et al., 2012), sarcopenia (Brioche and Lemoine-Morel, 2016), and inhibits protein synthesis by suppressing the AKT/mTOR pathway and its downstream targets (O'Loghlen et al., 2006, Tan et al., 2015, Zhang et al., 2009). Hence, It has been also reported that AMPK activation in response to oxidative stress inhibits protein synthesis through and Tuberous Sclerosis Complex 2 (TSC2) and mTOR1 phosphorylation (Zhao et al., 2017, Yoshida and Delafontaine, 2020).

The next important regulator of muscle atrophy is Myostatin, a secreted molecule of muscle-specific transforming growth factor-β (TGF-β) superfamily (Langridge et al., 2021, Mendias et al., 2012) (Figure 1.3). Myostatin acts to limit muscle growth by binding to its cell surface activin type II receptors, ActRIIβ/α leading to the activation of the Smad2/3 pathway (Sartori et al., 2009, Rodriguez et al., 2014, Huang et al., 2011, Sartori et al., 2021). Loss of myostatin in mice induces dramatic skeletal muscle hypertrophy (McPherron et al., 1997, Whittemore et al., 2003), while stimulation of skeletal muscle with myostatin induces wasting in-vivo and reduces myotube area in-vitro (Hoogaars and Jaspers, 2018, White and LeBrasseur, 2014, Trendelenburg et al., 2009). Some studies also indicate Smad transcription factors upregulate the expression of *Atrogin-1* and

MuRF-1 in skeletal muscle atrophy (Rodriguez et al., 2014, Zhou et al., 2010). Currently, Myostatin inhibition is pursued as a potential therapy for some myopathies (Lee et al., 2015).

As observed above, hypertrophy and atrophy occur as a response to a variety of stimuli factors that are fully linked to the health of skeletal muscle. In the next section, I specifically discuss the Hippo pathway and its role in skeletal muscle structure and function.

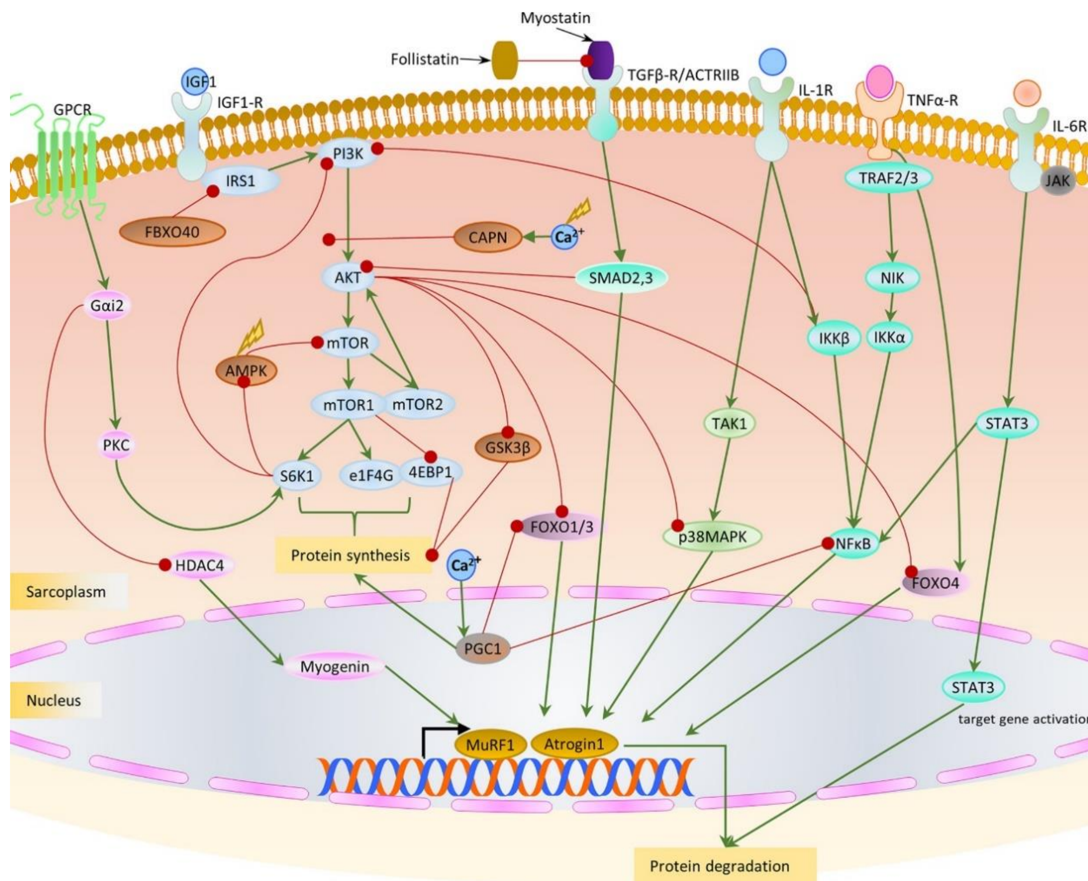

Figure 1.3. Signaling pathways controlling skeletal muscle mass.
Schematic representation of the major receptors and signaling pathways/proteins involved in atrophy and hypertrophy. The IGF/AKT pathway forms a key pathway for hypertrophy in muscle. While activation of MURF1/Atrogin1 via the SMAD, NFκB and STAT signaling lead to atrophy. Figure adapted from (Mukund and Subramaniam, 2020).

1.8 Hippo signal transduction network

Under physiological conditions, tissue homeostasis is mainly maintained through the precise regulation of cell apoptosis, differentiation, and proliferation (Meng, 2016). In the past two decades, the Hippo pathway has emerged as an evolutionary highly conserved signaling pathway that plays an essential role in the regulation of cell fate, tissue homeostasis, and organ size (Barry and Camargo, 2013, Piccolo et al., 2014, Varelas, 2014). In mammals, the canonical Hippo pathway consists of a kinase cascade of Mammalian sterile 20-like kinases 1 and 2 (MST1/2), and large tumor suppressors 1 and 2 (LATS1/2) (Gabriel et al., 2016). Active MST1/2 phosphorylate Mps One Binder Kinase Activator-Like 1 A/B (MOB1A/B) and Salvador Family WW Domain Containing Protein 1 (SAV1)(Callus et al., 2006, Praskova et al., 2008) two scaffold proteins that help MST1/2 in the phosphorylation and recruitment of LATS1/2 (Hergovich et al., 2006, Yin et al., 2013). Neurofibromin 2 (NF2) is another key player, which directly interacts with LATS1/ 2 and facilitates phosphorylation of LATS1/2 via the SAV1– MST1/2 complex (Yin et al., 2013). Once activated, LATS phosphorylates the main effectors of the Hippo pathway, Yes-associated protein (YAP), and transcriptional coactivator with PDZ-binding motif (WWTR1 or Taz)(Zhao et al., 2007a). LATS kinase activity inhibits YAP/TAZ transcriptional activity via changing YAP/TAZ localization and protein stability. Phosphorylated YAP/TAZ is sequestered in the cytoplasm by binding to 14-3-3, where they are ubiquitinated and degraded (Liang et al., 2014, Zhao et al., 2007b). On the contrary, when the LATS1/2 is inactive, dephosphorylated YAP/TAZ translocates to the nucleus to initiate transcription (Ren et al., 2010, Oh and Irvine, 2008). YAP and TAZ as transcriptional co-activators, do not have their DNA-binding motifs (Liang et al., 2014) thus they regulate gene expression through interaction with TEA Domain Transcription Factor 1-4 (TEAD1–4), which mediate the main transcriptional output of the Hippo signaling pathway in the cell (Zhao et al., 2008). Moreover, the Vestigial-like family members (VGLL1–4) have TDU motifs that can bind to TEAD1–4 and act as transcriptional cofactors for TEADs (Zhou et al., 2016) and may compete for common binding interfaces with Yap and Taz (Hori et al., 2020, Yamaguchi, 2020, Koontz et al., 2013). This suggests that VGLL proteins integrate into the wider Hippo signal transduction network (Hori et al., 2020, Yamaguchi, 2020, Koontz et al., 2013). YAP \TAZ also binds and coactivates transcription factors other than TEADs such as T-box transcription factor 5 (TBX5), and Sma gene mothers against the decapentaplegic peptide (Smad) (Ferrigno et al., 2002, Murakami et al., 2005) which are especially relevant to skeletal and cardiac muscle biology (Wackerhage et al., 2014). However, the roles of

these transcription factors (Smad and TBX5) in mediating the growth-promoting activities of YAP/TAZ have not yet been well established.

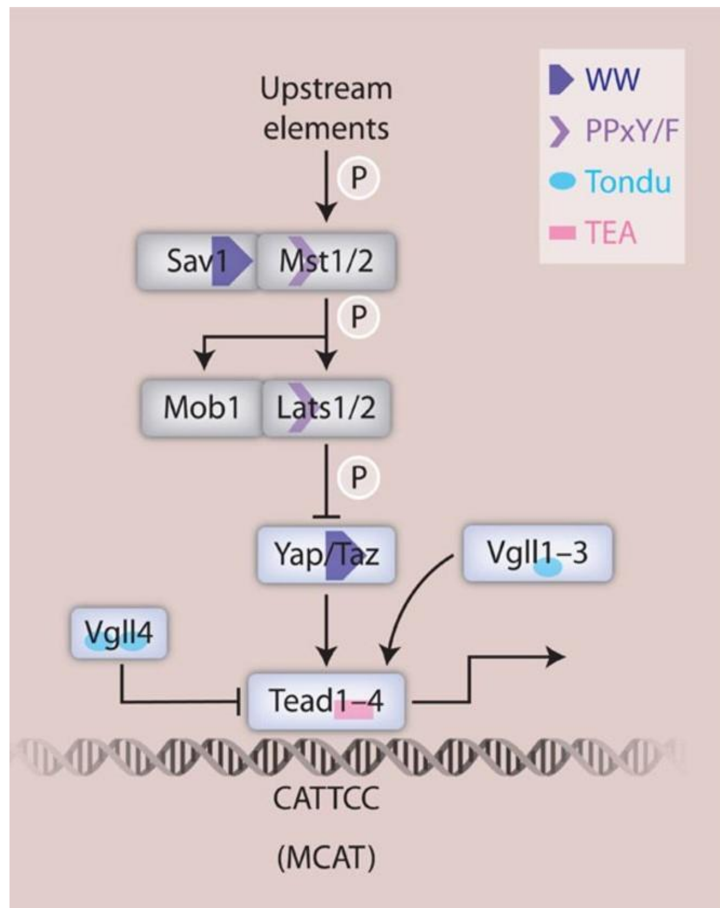

Figure 1.4 the Hippo signal transduction network.
Schematic of the Hippo signal pathway. The figure was adapted from Wackerhage et al (Wackerhage et al., 2014).

1.9 Hippo signal transduction network in skeletal muscle

The Hippo signal transduction network responds to various signals including cell polarity, cell-cell adhesion, hormones, mechanical cues, and cellular stress (Ma et al., 2019) and regulates the proliferation, differentiation, and size of satellite cells, myoblasts, and muscle fibers (Gabriel et al., 2016, Wackerhage et al., 2014). Additionally, previous evidence suggests that most of the major members of the Hippo pathway are expressed in the skeletal muscle (Watt et al., 2010).

Given the regulatory role of the hippo pathway in the determination of cell growth and the presence of the hippo proteins in the skeletal muscle, it has been a great interest for deep knowledge of molecular mechanisms underlying physiology, pathology, and treatment in skeletal muscle (Sun et al., 2017, Figeac et al., 2019, Judson et al., 2013, Feng et al., 2019).
To examine this, Judson et al developed an inducible skeletal muscle fiber-specific knock-in mouse model (MCK-tTA-hYAP1 Ser^{127A}) to test whether the overexpression of constitutively active Yap (hYAP1 Ser^{127A}) is sufficient to stimulate muscle hypertrophy or changes in fiber type composition. Unexpectedly, after five weeks muscle tissue-specific hYAP1 S^{127A} knock-in showed a reduction in muscle weight, muscle atrophy, and degeneration(Judson et al., 2013). On the contrary, the total Yap over-expression induced hypertrophy through interaction with TEADs family and independently of any mTOR pathway activity in post-mitotic adult skeletal muscle fibers (Watt et al., 2015).
Furthermore, It was found that the repression of *Yap* caused a reduction in the size of muscle fiber and muscle mass (Watt et al., 2015). Thus, it was concluded that the *Yap* expression would be necessary for increasing and maintaining the muscle fiber size and muscle mass. Moreover, Goodman et al found that Yap is up-regulated by mechanical overload and is sufficient to induce skeletal muscle hypertrophy. Imposing a mechanical overload to the plantaris muscle by agonists ablation was associated with a higher expression of *Yap*, thereby highlighting *Yap* sensitivity to mechanical loading on the skeletal muscles (Goodman et al., 2015).

In another study, MST1 was identified as a key player in enhancing fast-twitch muscle fiber atrophy. MST1 kinase mediates the pathologic process of neurogenic muscle atrophy via direct phosphorylation of the forkhead box O-3 a (FOXO3a) transcription factor at Ser^{207}, and activation of atrogenes. In addition, the Loss of *Mst1* results in a higher slow type I myofibers in the soleus (Wei et al., 2013).

A study showed another primary downstream effector of the Hippo pathway, Taz, plays important role in the proliferation and regeneration of muscle cells. They reported Taz operates through Tead4 to control myogenic differentiation. Also, the absence of *Taz* results in a greater proportion of slow type I myofibers (Sun et al., 2017).

Evidence suggests that Vgll family members also play an important role in the skeletal muscle. *Vgll2* for instance is highly expressed in skeletal muscle and involved in the differentiation of muscle cells (Chen et al., 2004). It also has a key role in muscle fiber-type distribution. Honda et al revealed that the absence of *Vgll2* leads to a higher number of fast-twitch type 2b (IIb) fibers and downregulation of slow isoform MyHC gene (*Myh7*) and its intronic miRNA (miR-208b), thereby

shifts in the slow to fast muscle fiber type (Honda et al., 2017). Furthermore, another Vgll family member, Vgll3 has been identified as a transcriptional co-factor operating with the Hippo signaling pathway to regulate myogenesis. *Vgll3* knockdown suppressed the proliferation of myoblasts, whereas *Vgll3* over-expression strongly promoted muscle cell differentiation (Figeac et al., 2019). Evidence also suggests that *Vgll4* homozygote knockout leads to smaller muscle mass, myofiber size, and functional defects (Feng et al., 2019).

Tead transcriptional factors as another hippo pathway members have a great role in muscle gene expression. Previous data show that *Tead1* is a sufficient factor in either muscle-specific gene expression and regeneration (Tsika et al., 2008, Southard et al., 2016). It also has been reported that *Tead1* overexpression induces satellite cell hyperplasia, and a transition toward a slow-twitch muscle fiber phenotype (Tsika et al., 2008, Southard et al., 2016). Furthermore, Benhaddou et al showed that Teads activity is essential for normal C2C12 cell differentiation. Hence, Tead4 regulates the myogenin expression during cell differentiation in C2C12. It is also important for myoblast fusion because of inducing myogenin via binding MCAT elements in its promoter (Benhaddou et al., 2012).

Despite the previous studies that indicated the role of Hippo signaling in skeletal muscle, there is much yet to learn regarding the role of the Hippo members in skeletal muscle. Therefore, I aimed to study the role of the Hippo-related genes in skeletal muscle in this Ph.D. project.

1.10 Aim of the thesis

The genetics of many physiological and pathological skeletal muscle-related traits are still incompletely understood. To address a part of this gap in our knowledge of the genetics of skeletal muscle trait variation, I conducted two studies:

1) In the first study, I report the results of a systematic review on the genes whose gain or loss-of-function increases endurance capacity in mice. Additionaly I studied the identified endurance genes further through bioinformatical analyses.
2) In the second study, I describe and discuss the results of phenotyping the hindlimb muscles of mice with mutations of genes of the Hippo signal transduction network (i.e. *Lats1*$^{-/-}$, *Vgll3*$^{-/-}$, *Vgll4*$^{+/-}$, and *Mst2*$^{-/-}$).

Chapter 2 Methods

2.1 Systematic reviews

2.1.1 Systematic literature search

2.1.1.1 Search strategies for systematic reviews

I conducted a systematic reviews using the PRISMA guidelines (Moher et al., 2009) and included all studies according to the participants, interventions, comparators, and outcomes (PICO) process (Schardt et al., 2007). Its main aim was to identify genes whose gain or loss-of-function results in improved endurance capacity in mice.

2.1.1.2 Search strategy to identify genes whose transgenesis improve endurance capacity

I first searched the six English-language databases (Google Scholar, Bio-Med, Scopus, PubMed, Science Direct, and Web of Science) using our systematic search strategy and used the following combination of search terms: ("mouse" OR "murine" OR "mouse model" OR "mice" OR "mice transgenic") AND ("overexpression" OR "knock out" OR "knock-in" OR "gene transfer techniques" OR "mutagenesis" OR "gene deletion" OR "gene manipulation") AND ("endurance exercise" OR "swimming" OR "wheel running" OR "endurance capacity" OR "mPXT" (speed progress until exhaustion test in mice) OR "mGXT" (graded maximal exercise in mice)).

2.1.2 Inclusion and Exclusion criteria

After eliminating duplicates, we examined the published studies in two stages: First, we reviewed results by title and abstract and then by full-text. At each step, we deleted studies that did not match with the inclusion and exclusion criteria of studies. We included studies in this systematic review if they met the following criteria:

Table 2.1 Inclusion and exclusion criteria

Endurance capacity
No wild-type mice as a control group
No transgenesis, double mutation, or long non-coding RNA manipulation Major pathological abnormalities result from the gene manipulation
Rat or *in vitro* study
No statistically significant effect or no outcome measures
Mice are older than 24 months
Use of an additional drug treatment or dietary supplement (we included studies where a transgene was induced, e.g., through doxycycline injection)

2.1.3 Data Extraction

We extracted the following information from each relevant study that met the exclusion/inclusion criteria (Table2.2). Sometimes, the output measure values were presented only in a bar graph and not as a number. In these cases, we manually measured and estimated the relative difference of mean values between controls and transgenic mice by using the bar height. Moreover, we adopted official gene names from the Universal Protein Resource (UniProt, NCBI) and the official gene names may differ from the gene or protein names that are used in the original papers.

Table 2.2 Data Extraction

Endurance capacity
Author
Gene name
Protein name
Method of transgenesis
Acclimated to exercise
Exercise testing protocols
Output measure (distance, time, maximal speed)
Relative difference outcome measures between transgenic and control mice
Age of mice
Mouse strain
Remarks (Supplementary Table S2)

2.1.4 Bioinformatical analyses

2.1.4.1 Bioinformatic analysis for systematic review

To obtain more information about the identified endurance genes performed bioinformatical analyses. For each review that I conducted common but also distinct bioinformatical analysis. This included the use of web-based tools or comparison with publicly available datasets.

To obtain more information about the identified endurance genes and the proteins that they encode, we asked several research questions and performed bioinformatical analyses to answer these questions. The research questions and analyses for each systematic review were as follows:

Table 2.3 Research questions and data source & extraction for endurance systematic review

Research questions	Data source and extraction
Do endurance genes overlap with DNA variants that are associated with human endurance?	Comparison with the list of human genes where DNA variants associated with endurance (Ahmetov et al., 2016)
Do endurance genes overlap with DNA variants that are associated with VO2max?	Comparison with the list of human genes where DNA variants associated with VO2max trainability (Williams et al., 2017)
Do endurance genes overlap with DNA variants that are associated with endurance related traits?	GWAS catalog (MacArthur et al., 2017; https:// www.ebi.ac.uk/gwas/; RRID:SCR_012745)
In what tissues are the endurance genes expressed?	Genotype-Tissue Expression (GTEx; RRID:SCR_001618; (GTEx Consortium, 2015).
Are endurance genes regulated by acute endurance and/or resistance exercise?	GSE59088 (Vissing and Schjerling, 2014) from Gene Omnibus (https://www.ncbi.nlm.nih.gov/geo/; RRID:SCR_007303)
Are endurance genes phosphorylated or dephosphorylated in response to exercise?	Hoffman et al., 2015 supplementary Table S1
Do endurance genes interact?	STRING database analysis (Szklarczyk et al., 2015; https://string- db.org/; RRID:SCR_005223)
Do endurance genes have common functions?	Enrichment analysis using ToppGene (RRID:SCR_005726; Chen et al., 2009)
How many proteins encoded by endurance genes are predicted to be secreted in blood?	Secreted proteins from the Human Protein Atlas (https://www.proteinatlas.org/; Uhlen et al., 2015) were compared with endurance genes.

The data I obtained to answer the above research questions, I have collected in spreadsheets or plotted in figures, which are included in this thesis or my published paper.

2.2 Methods for experimental study

2.2.1 Ethical approval and Husbandry

All animal procedures include ethical statements and housing & husbandry condition that are available on the IMPC portal (http://www.mousephenotype.org/about-impc/arrive-guidelines).

2.2.2 Mouse generation and phenotyping

To study the role of the Hippo related genes in skeletal muscle, the hindlimbs of global knockout mice include *Lats1*$^{-/-}$ (*Lats1*$^{em1(IMPC)H}$), *Mst2*$^{-/-}$ (*Stk3*$^{em1(IMPC)H}$), *Vgll3*$^{-/-}$ (*Vgll3*$^{em1(IMPC)H}$), and *Vgll4*$^{+/-}$ (*Vgll4*$^{tm1b(EUCOMM)Hmgu}$) were kindly supplied by the Helmholtz Zentrum München (HMGU) in Germany, and the Medical Research Council Harwell (MRC Harwell) in the UK. Mice were generated on C57BL/6N background and phenotyping data were collected at the age of 16 weeks. For each of the hippo-related genes knockout lines, at least three hind limbs were collected at regular intervals on age and sex-matched control mice of equivalent genetic backgrounds. As detailed in the allele design for Knockout mice (https://www.mousephenotype.org/understand/the-data/allele-design/), Phenotyping Process (https://www.mousephenotype.org/understand/the-data/phenotyping-process%20impress/), and data output presented on IMPC portal (http://www.mousephenotype.org/).

To phenotype the hindlimb muscles of Hippo gene-mutated mice and their matched control, we first dissected the hindlimb muscles of mutated and control mice. The analyzed muscles are the tibialis anterior (TA), extensor digitorum longus (EDL), gastrocnemius, and soleus. After dissection, we weighed the hindlimb muscles and then calculated the relative muscle weight (muscle weight/whole body weight).

2.2.3 Cell culture

Mouse C2C12 myoblasts cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Cat#31885, Waltham, MA, USA), supplemented with 10% fetal bovine serum (Sigma, Germany), and incubated at 37 °C in humidified air with 5% CO2. To induce differentiation, the myoblasts were cultured in a growth medium until confluence, then the medium was switched to DMEM with 2% horse serum (Sigma, Germany). The Proliferation and differentiation medium was refreshed daily up to 96 (Figure 2.1). After 48-96 hours of differentiation medium, C2C12 Myotubes untreated (DMSO) or 24 h treated with exercise stimulates including 100 μM Clenbuterol (Sigma, Germany) to induce hypertrophy, or 1 mM 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; Cell Signaling Technology, USA) to induce energy stress.

2.2.4 RNA isolation, reverse transcription, and quantitative Real-Time PCR

Following the dissection of the mice's hind limb, the soleus muscle samples were homogenized in 1ml QIAzol (QIAGEN, Germany) using a Precellys lysing kit (Bertin Instruments, USA) and Precellys homogenizer machine (Bertin Technologies, USA) with high speed for 40 seconds. For C2C12 Myotubes, after washing cells with phosphate-buffered saline (PBS), cells were lysed in lysis T buffer (Peqlab Biotechnology GmbH, reference number 12-6634-01) and stored at -80 °C. The total RNA was extracted with the peqGOLD Total RNA Kit C-Line (Peqlab Biotechnology GmbH, reference number: 12-6634-01) according to the manufacturer's instructions. RNA concentrations were determined by a Nanodrop spectrophotometer (ND-100 spectrophotometer; Nanodrop Technologies, Wilmington, DE, USA). RNA purity was ensured by a 260/280 ratio (range 2.00–2.11, mean 2.04). For mRNA analysis, cDNA was synthesized using 2 μg of total RNA and qScript XLT cDNA SuperMix (Quantabio, product number 030256) as specified by the manufacturer. cDNA was amplified, using a PerfeCTa qPCR fluorescent SYBR Green SuperMix (Quantabio, product number: 023916), with a real-time quantitative PCR (Rotor-Gene RG 6000 - QIAGEN). The primer sequences used in this study were as follows: *Lats1*: forward (F) 5′-AATGAAATGATGCGGGTTGGA -3′ and reverse (R) 5′- CAGACTTCACCAAACGCTCC -3′; *Rpl7*: (F) 5′-ACGGTGGAGCCTTATGTGAC-3′ and (R) 5′-TCCGTCAGAGGGACTGTCTT-3′. Transcriptional expressions of the target genes were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and referenced to the Rpl7 housekeeping gene (Thomas et al., 2014).

2.2.5 Western blotting

To extract the total protein, isolated soleus muscles from each mouse was homogenized in lysis buffer (0.1% SDS,0,5 M sodium -orthovanadate, 0.5% sodium deoxycholate, 50 mM NaF, 1 mM EDTA,150 mM NaCl, 1%Triton-X 100) supplemented with protease and phosphatase inhibitor cocktail (Peqlab Biotechnology GmbH, Germany) using a Precellys lysing kit (Bertin Instruments, USA) and precedes homogenizer machine (Bertin Technologies, USA) with high speed for 40 seconds. After centrifugation at 12,000 g for 10 minutes at 4 °C, the supernatant was transferred to a new tube and assayed to determine the protein concentration of each sample using the Bradford protein assay kit (BioRad Laboratories GmbH, Cat#5000111, Munich, Germany). Homogenates of each muscle sample were diluted to a protein concentration of 1 mg/ml using a 4× SDS sample buffer (0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 20% β-mercaptoethanol, and 0.05% bromophenol blue). Samples then were heated at 95 °C for 5 min, and 30 µg of whole protein lysates were subjected to 10% SDS-PAGE gel (Bio-rad, Germany). Proteins in the gels were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-rad, Germany) using a Trans-Blot Turbo Blotting System (Bio-Rad, Germany). Following a blocking step (5% non-fat milk powder, 1X Tris-buffered saline,1% Tween-20) the PVDF membrane was incubated overnight with primary antibody and probed with the appropriate horseradish peroxidase (HRP)-linked secondary antibodies for 1h at RT. The membranes were developed with enhanced chemiluminescence (ECL) (Bio-Rad, Germany), and the signals were detected by an INTAS Chemocam Imager (Royal Biotech GmbH, Germany). The antibodies used in this study were as follows: Anti MyHC-I (1:1000, DSHB Cat# BA-D5, RRID: AB_2235587) and Anti-mouse IgG, HRP-linked Antibody (1:2500, Cell Signaling Technology Cat# 7076, RRID: AB_330924). Quantification of immunoreactive bands was performed using ImageJ software (http://rsb.info.nih.gov/ij/index.html) and the largest bond of Ponceau S staining was used for normalization.

2.2.6 Muscle cryosectioning and histology

The tibialis anterior (TA) and soleus muscle of mice were removed, frozen in isopentane (2-methyl butane) cooled with liquid nitrogen, and sectioned in a microtome cryostat (LEICA CM3050 S) and mounted on SuperFrost® slides (VWR International GmbH, Germany Cat#631-1349) air-dried and stored at -80 °C until further analysis.

2.2.7 Haematoxylin & Eosin histochemical stain

To show whether Hippo mutated genes affect the cytoplasmic, nuclear, and extracellular matrix structures of mice skeletal muscle, I performed the hematoxylin and eosin (H&E) stain. To perform H&E staining, the frozen soleus cryosections (10 µm) were thawed at room temperature. The cryosections first were rinsed in distilled water, incubated in filtered 0.1% Mayers Hematoxylin (Carl Roth, Germany, Cat#T865.3), for 5 minutes, and then rinsed in tap water. The slides were then incubated in 0.3% acid alcohol (1% Hydrochloric acid in 70% Etanol) three times, then rinsed in tap water, and placed in lithium carbonate for 30 seconds. Following further washes in tap water, slides were finally stained with 1% eosin Y solution (Carl Roth, Germany, Cat#X883.2) for 1 min.

2.2.8 NADH tetrazolium reductase histochemical stain

To stain highly oxidative (typically corresponding to type I and IIa fibers) and less oxidative fibers (corresponding to type IIx and IIb fibers), I used an NADH tetrazolium reductase (NADH-TR) histochemical reaction. For this, frozen TA muscle cryosections (10 µm) were thawed at room temperature and incubated with NADH-TR staining solution (2.52 g Tris HCl, 0.10 g Nitro blue tetrazolium, 0.68 g Tris base, dissolved in 100 ml distilled H_2O -1 mg of NADH added to 1 ml staining solution (pH: 7.4) before staining) for 30-60 minutes in a humidified chamber at room temperature. Samples were washed in distilled water before the dehydration step.

2.2.9 Adenosine triphosphatase (ATPase) histochemical stain

To differentiate in-between type I and type II muscle fibers I used an ATPase histochemical reaction with an acid preincubation to selectively stain type I fibers brown-black. For this, 10 µm soleus sections were thawed at room temperature for 5 min and pre-incubated in an acetate buffer at pH 4.47 for 10 min followed by an Adenosine 5'-triphosphate reaction solution for 30 min at 37 °C then rinsing in distilled water. The slides were then incubated in 1% Calcium chloride for 3 min then rinsed in distilled water and placed in 2% cobalt chloride solution for 3 min. Following further washes in distilled water, slides were finally incubated in a 1% ammonium sulfide solution for 1 min and washed 3 times in distilled water.

2.2.10 Dehydration and mounting

All sections from H&E staining, NADH-TR, and ATPase enzyme activity analysis were dehydrated by sequential dipping in 70% and 100% ethanol and then ROTI Histol (Carl Roth, Germany), before being mounted with coverslips using a DPX mounting medium.

2.2.11 Microscopy

Digital photos of cross-sections were captured in 2.5, 5, and 20-X magnifications using a Zeiss Axio Lab.A1 equipped with a digital camera (Zeiss, Germany) and Zeiss ZEN Software version 2.6 (Blue edition; Zeiss, Germany).

To determine the cross-sectional area (CSA), Twenty-five myofibers per fiber type, per sample were analyzed. To distinguish fiber types (type I and II) percentages of muscle fiber types were quantified manually by counting stained and unstained fibers (5-fold magnification). All microscopy analysis was performed using the ImageJ software (http://rsb.info.nih.gov/ij/index.html), RRID: SCR_003070).

2.2.12 Re-Analyses of published datasets

To investigate whether diseases or other stimuli *Lats1* expression I retrieved several gene expression datasets from Gene Expression Omnibus (GEO). These include the dataset (GDS4924) for the regeneration after cardiotoxin-induced muscle injury in TA muscle of mice (Lukjanenko et al., 2013), the transcriptome dataset (GSE23244) for comparison fiber type I and IIb in mice muscle (Chemello et al., 2011), microarray dataset GDS4932 for muscle hypertrophy after synergist ablation-overloaded in plantaris muscle of mice (Chaillou et al., 2013), and microarray dataset (GDS609) for muscle dystrophy in quadriceps muscle of Duchenne muscular dystrophy (DMD) patients and unaffected controls (see the supplementary data S1A in Haslett et al., 2003)(Haslett et al., 2003a). I also investigated whether the *Lats1* gene changes its expression after exercise training. For this, I downloaded supplementary data files from studies comparing resting, 2.5, and 5hrs endurance exercise or resistance (strength) exercise-trained human skeletal muscle (Vissing and Schjerling, 2014b). Lastly, to obtain a forest plot of the expression of *Lats1*gene in response to acute aerobic and acute resistance exercise I re-analysis the meta-analysis of all available human skeletal muscle studies. The original data was obtained from Meta MEx Portal (http://www.metamex.eu/) (Pillon et al., 2020).

2.2.13 Statistical analysis

To analyze the normal distribution of data I performed the Kolmogorov - Smirnov (K-S) normality test. Then, I analyzed the data by an Unpaired t-test to determine significant differences between conditions. Results are expressed as mean ± standard error (SEM) and considered significant at $P<0.05$. All statistical analyses were performed using Prism version 8.0 statistical software package (GraphPad Prism; RRID: SCR_002798).

Chapter 3 Results

Part 1 – Genes that regulate endurance capacity

Publication 1
Authors: Yaghoob Nezhad, F., Verbrugge, S. A.J., Schönfelder, M., Becker, L., Hrabě de Angelis, M.,& Wackerhage, H. **Title**: Genes whose gain or loss-of-function increases endurance performance in mice: a systematic literature review. **Journal:** Frontiers in Physiology
DOI: 10.3389/fphys.2019.00262

3.1.1 Summary

Genetics for decades has been highlighted to associate with a lot of exercise-related traits and phenotypes including endurance performance. Twin and family studies suggest that several key endurance-associated variables such as VO_2max, VO_2max trainability, and muscle fiber-type distribution are ≈50% inherited. In addition, human genome-wide association studies (GWAS) and whole-genome sequencing (WGS) studies try to target and identify specific variants or all genetic variations (respectively) that are associate with endurance-related traits. Forthemore, researchers study the candidate genes/DNA sequences that may affect endurance-related variants in transgenic living organisms such as mice models. The most remarkable example was the overexpression of the PEPCK enzyme(*Pck1*) in mice's skeletal muscle which could highly enhance endurance performance. While GWAS, WGS and transgenic living organisms studies have identified some causal genes and DNA variants, much still needs to be discovered. In this systematic review, I collected the existing evidence to answer the gain or loss of function of what genes increase endurance performance. I identified 31 genes whose manipulation increase mice endurance performance by up to 1800% when compared to the control mice. Further bioinformatical analyses reveal the DNA sequence variability of these genes in humans, their organ-specific expression pattern, functional links in-between these genes and the proteins they encode, and a role for some endurance genes during adaptation to endurance exercise. The endurance gene list also provides an up-to-date candidate list for more targeted human genetic analyses for endurance performance or trainability.

This study as an original systematic literature review was accepted on the 28th of February 2019 and published on the 22nd of March 2019 in “Frontier in physiology” journal by Fakhreddin Yaghoob

Nezhad, Sander Verbrugge, Martin Schönfelder, Lore Becker, Martin, Hrabe de Angelis, and Henning Wackerhage.

Contribution

I am the first author of this paper and conducted the systematic literature research, collected data from publications, analyzed data, and performed bioinformatics analyses. Henning Wackerhage conceptualized the idea behind this manuscript. Together with Henning Wackerhage and Sander Verbrugge, I wrote the first draft. all other authors and I contributed to the final submitted manuscript.

Systematic literature review

Based on the search strategy, we screened 2315 manuscripts with publication dates until January 2018. regarding the title or abstract, we excluded 2171 studies. After that, 263 articles remained which we read fully for eligibility. we identified another 43 articles by reviewing the reference lists of the full-text articles or other sources. Finally, we read 144 full-text articles and analyzed 32 articles quantitatively. A PRISMA flowchart of our search and reading strategy is in Figure 3.1. I used the information from this systematic literature search and several bioinformatical analyses to answer several research questions which are stated as headers below.

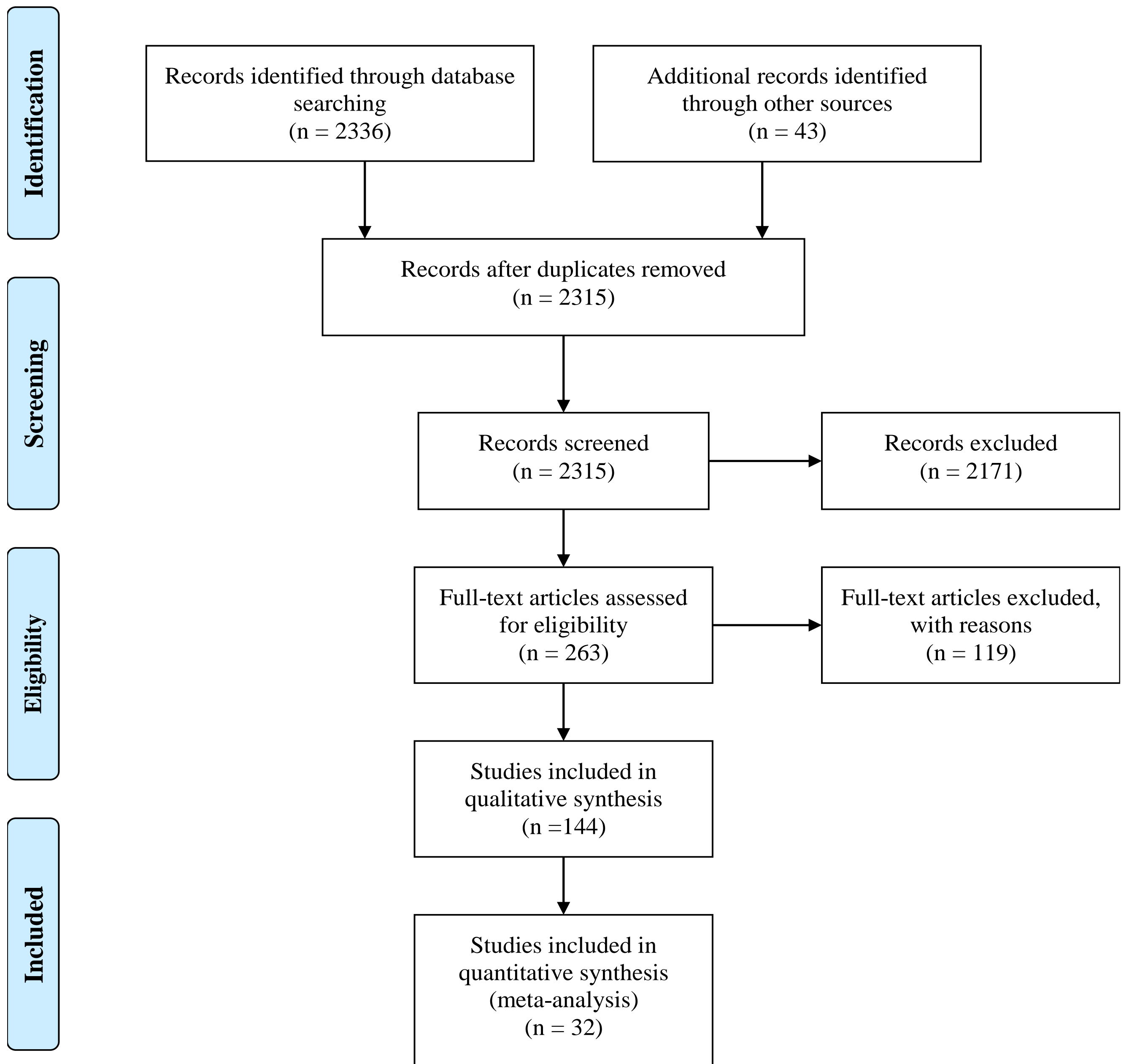

Figure 3.1 PRISMA flowchart on search and selection of eligible articles.

3.1.2 The gain or loss-of-function of what genes increase endurance performance?

My analysis revealed 31 genes/isoforms including two isoforms of *Ppargc1a* whose gain or loss-of-function increased endurance performance in mice. Specifically, I identified 19 genes (*Adcy5, Adcy8*, *Hk2, , Mef2c, Nr4a3, Pck1* (Pepck), *Ppard, Ppargc1a* (both the a and b isoforms of the mitochondrial biogenesis regulator Pgc-1α), *Ppargc1b, Ppp3ca* (calcineurin)*, Scd1, Slc5a7, Tfe3, Tfeb, Trib3* & *Trpv1;* Figure 3.1) whose gain-of-function increased endurance capacity in mice. I also found 14 genes (*Actn3, Adrb2, Bdkrb2, Cd47, Crym, Hif1a, Myoz1, Pappa, Pknox1, Pten, Sirt4, Thbs1, Thra* & *Tnfsf12*; Figure 3.2) whose loss-of-function increases endurance capacity. Collectively, I will refer to these genes as endurance genes for simplicity. The relative increase ranged from 12% for *Pten* to 1800% for *Pck1* (Figures 3.1 and 3.2). To explain, if the mean value for the wildtype mouse was e.g. 100 units in an endurance test, then the transgenic mice achieved on average between 112 units (i.e. an increase of 12 units or %) or 1900 units (i.e. an increase by 1800 units or %), respectively. The detailed experimental design and endurance performance data for each transgenic and wild-type mouse pair are summarized in table S.1 (see Appendix) and in full detail in supplementary data S2.

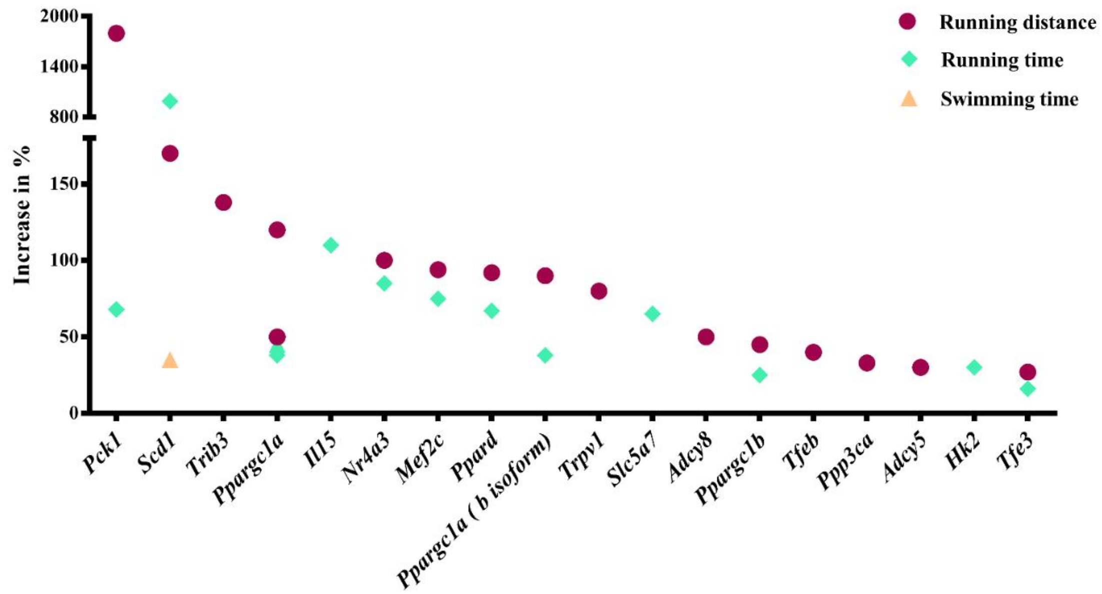

Figure 3.2. Genes whose gain-of-function increases running or swimming endurance in mice.
Percentage increase in % was calculated by direct comparison the control animals. Finally, genes are plotted from high to low effect size.

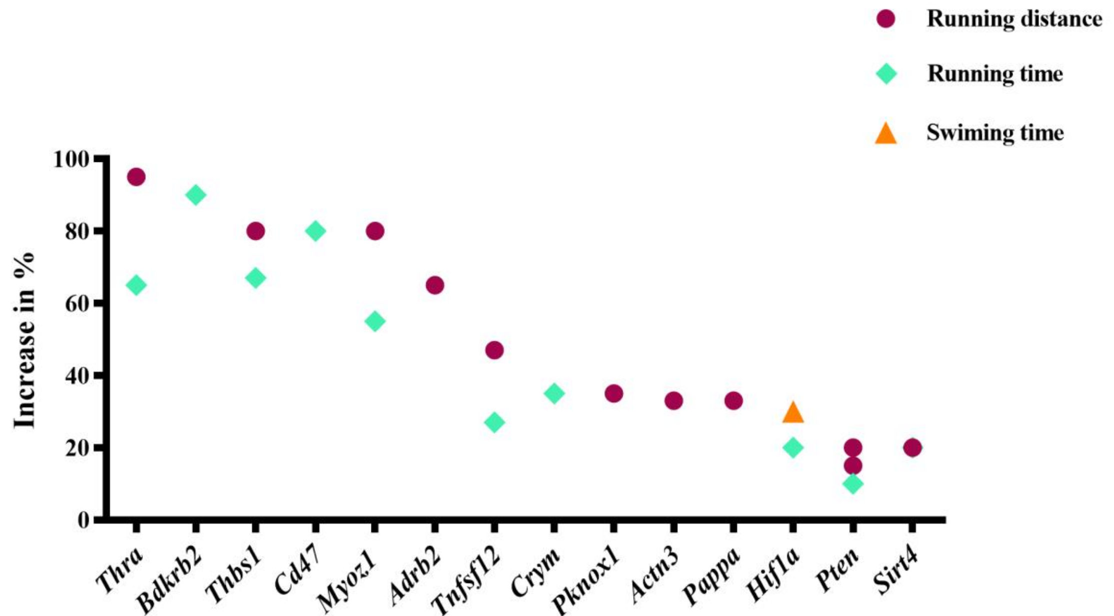

Figure 3.3. Genes whose loss of function increases running or swimming endurance in mice.
Percentage increase in % were calculated by direct comparison the control animals. Finally, genes are plotted from high to low effect size

3.1.3 Do endurance genes overlap with human genes where DNA variants are associated with human endurance, VO_2max trainability and other endurance-related traits?

To study whether endurance genes also play a role in the variation of human endurance, I overlapped our set of endurance genes with a list of human genes where DNA variants associated with endurance (Ahmetov et al., 2016) and genes where DNA variants associate with VO_2max trainability (Williams et al., 2017). Moreover, I searched for phenotypes for endurance genes reported in human genome-wide association studies (GWAS) by searching the human GWAS catalog. The first overlap analysis I screened the human homologs of the mouse genes. Here, we revealed that human endurance gene variants of *ACTN3*, *ADRB2*, *BDKRB2*, *HIF1A*, *PPARD*, *PPARGC1A*, *PPARGC1B,* and *PPP3CA* are also associated with human endurance (Ahmetov et al., 2016). Moreover, DNA variants linked to *ADCY5*, *PPARD,* and *HIF1A* are associated with VO_2max trainability in humans (Williams et al., 2017). In a second step, I investigated whether the identified endurance genes were linked in genome-wide association studies (GWAS) to phenotypes that are potentially relevant for endurance performance. To do so, I performed a GWAS catalog

search for each gene. This search revealed several associations between endurance genes and several physiological and pathological human phenotypes (supplementary data S3). Associations of potential relevance for endurance performance include an association of *PCK1* with the hemoglobin concentration (Astle et al., 2016), an association of *PPARGC1A* with the resting heart rate (Eppinga et al., 2016), an association of *SCD* with metabolic traits (Suhre et al., 2011) specifically blood levels of myristate (14:0) / myristoleate (14:1n5) (Shin et al., 2014)), and an association of *TFEB* with left ventricular wall thickness (Wild et al., 2017). Collectively, these analyses demonstrate that some endurance genes are associated with human endurance-associated traits, too.

3.1.4 How much does the DNA sequence of human endurance gene exomes vary in 60,706 humans?

Next, I used the ExAC browser to explore the extent to which the DNA sequence of human endurance gene exoms varies (Lek et al., 2016). This analysis revealed extensive genetic variation of endurance genes in humans. On average, each human homolog of an endurance gene had 174 missense DNA variants, 5 loss-of-function DNA variants and 11 copy number DNA variants. Additionally, for ACTN3, CRYM, TFE3, and THRA homozygous loss-of-function DNA variants were reported. For ACTN3 it is already known that a loss-of-function can be tolerated as ≈20% of the population are homozygous for an ACTN3 R577X variant (Yang et al., 2003). This and the results of genome-wide association studies suggest a large amount of common or rare, functionally relevant DNA sequence variation in the human homologs of mouse endurance genes.

3.1.5 In what human tissues are endurance genes expressed?

I have already mentioned that endurance is a trait that is determined by the function and interplay of several organ systems. These include skeletal muscle as the key force-generating and energy converting organ, the liver for glycogen storage and gluconeogenesis, the oxygen-delivering organs lung, heart, vasculature, and blood as well as the brain due to its role in mental fatigue. To study the expression in resting human organs, I retrieved gene expression data from the GTEx Portal database (GTEx Consortium, 2015) and plotted this as a heat map (Figure 3.3, supplementary data S5). This reveals that *Myoz1* and *Actn3* are selectively expressed in skeletal muscle whereas *Pck1* is selectively expressed in the liver, at least at rest. In addition, several other genes, such as *Sirt4, Ppargc1a, Il15, Adcy8, Bdkrb2, Pappa,* and *Slc5a7*, are not expressed in these selected organs.

Gene	Skeletal muscle	Liver	Lung	Heart (left ventricle)	Artery (aorta)	Whole blood	Brain (cortex)	Name
MYOZ1	1875	0	0	0	0	0	0	Myozenin1
ACTN3	123	0	0	0	0	0	0	Actinin alpha 3
MEF2C	45	0	15	6	38	3	53	Myocyte enhancer factor 2C
TNFSF12	34	12	86	21	173	29	53	TNF superfamily member 12
THRA	29	0	34	18	55	5	97	Thyroid hormone receptor alpha (p43)
TFEB	25	0	15	9	14	39	6	Transcription factor EB
TFE3	23	21	95	15	98	110	55	Transcription factor E3
HK2	21	0	22	4	12	37	0	Hexokinase 2
PPP3CA	17	0	24	0	18	13	92	Calcineurin
NR4A3	15	0	13	3	9	0	0	Nuclear receptor subfamily 4 group A member 3
PPARD	15	13	43	8	34	13	37	Peroxisome proliferator activated receptor delta
PPARGC1A	12	9	3	10	0	0	5	PPARG coactivator 1 alpha
PTEN	10	12	34	9	37	46	12	Phosphatase and tensin homolog
ADRB2	6	6	29	4	7	7	0	Adrenoceptor beta 2
CD47	5	6	62	7	41	15	27	CD47
PKNOX1	5	3	10	2	8	6	15	PBX/knotted 1 homeobox 1
SIRT4	4	3	2	3	2	0	3	Sirtuin 4
TRPV1	4	14	6	4	5	1	7	Transient receptor potential cation channel V1
PPARGC1B	3	1	5	4	0	0	2	PPARG coactivator 1 beta
IL15	1	1	5	1	5	1	0	Interleukin 15
ADCY5	0	0	7	40	70	0	27	Adenylate cyclase 5
ADCY8	0	0	0	0	0	0	6	Adenylate cyclase 8
BDKRB2	0	0	2	0	1	0	0	Bradykinin receptor B2
CRYM	0	0	0	32	0	0	71	Crystallin mu
HIF1A	0	15	132	17	78	71	20	Hypoxia inducible factor 1 alpha
PAPPA	0	0	2	0	4	0	0	Pappalysin 1
PCK1	0	838	0	0	0	0	0	Phosphoenolpyruvate carboxykinase 1 (Pepck)
SCD1	0	0	80	0	0	0	205	Stearoyl-CoA desaturase
SLC5A7	0	0	0	0	0	0	0	Solute carrier family 5 member 7
THBS1	0	0	446	0	109	22	0	Thrombospondin 1
TRIB3	0	35	17	1	5	0	0	Tribbles pseudokinase 3

Figure 3.4. Heatmap illustrating the expression levels of endurance genes in transcripts per million (TPM) in some endurance exercise organs.
If no expression value was given in GTEx Portal, I recorded the expression as “0” which signifies either no expression or no data

3.1.6 Do endurance genes change their expression in muscle after human endurance and resistance (strength) exercise? What endurance genes/proteins are detected as phosphorylated proteins at rest or after high intensity exercise in human muscle?

Several genes may be expressed at low levels in resting human organs but may increase their expression in response to endurance exercise. One example is the gene *Ppargc1a* which encodes various isoforms of the mitochondrial biogenesis regulator Pgc-1α. The expression of *Ppargc1a* increases in response to endurance exercise both in mouse (Baar et al., 2002) and human skeletal muscle (Pilegaard et al., 2003). Moreover, proteins encoded by endurance genes may become phosphorylated after a bout of endurance exercise as can be demonstrated by phosphoproteomics (Hoffman et al., 2015). To test whether endurance genes/proteins change their expression or phosphorylation after a bout of endurance exercise, I re-analyzed published datasets (Vissing and Schjerling, 2014a, Hoffman et al., 2015). These analyses reveal that *PPARGC1A* (encoding PGC-1α), *NR4A3* which encodes a nuclear hormone receptor, and *THSB1*, which encodes thrombospondin 1, are examples for genes that increase their expression 2.5 h and 5 h in the vastus lateralis after cycling for 120 min at 60% of the VO_2peak (Figure 3.4, supplementary data S6 (Vissing and Schjerling, 2014a). In contrast, *ACTN3* decreases its expression after human endurance exercise (Figure 3.4). Whilst the direction of the expression changes of *PPARGC1A*, *NR4A3* and *ACTN3* is consistent with the respective mouse phenotypes, *THSB1* expression increases after endurance exercise but a global deletion of *Thsp1* increases capillarity and exercise capacity in mice (Malek and Olfert, 2009). Thus, *THSB1* expression after endurance exercise suggests that it promotes a reduced adaptation to endurance

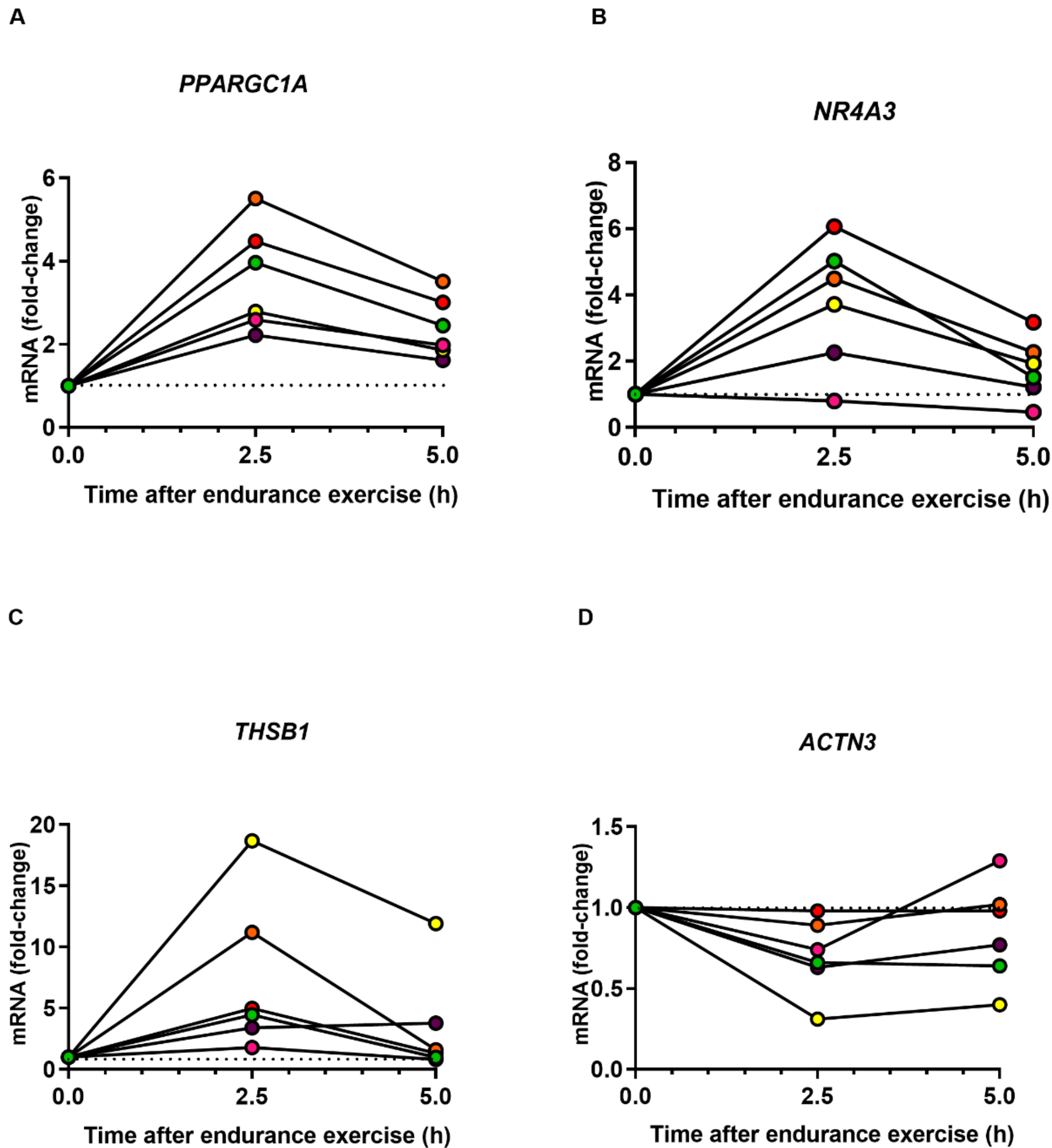

Figure 3.5. Effect of human endurance exercise on the expression of endurance genes in the vastus lateralis 2.5 and 5 h after exercise (Vissing and Schjerling, 2014a).

exercise. In addition, proteins encoded by *ACTN3, ADRB2, MEF2C,* and *TFEB* were detected as phosphorylated proteins in vastus lateralis samples after a single bout of high-intensity cycle exercise at ≈90% of the maximal power output (Wmax) for ≈10 min, (supplementary data S7). Of these proteins, TFEB phosphorylation significantly decreased from pre to post-exercise (Hoffman et al., 2015). Collectively these expression and phosphoproteomics data show that some

endurance genes change their expression or phosphorylation in human skeletal muscle after exercise and are therefore potential regulators of skeletal muscle endurance adaptations.

As I have mentioned, skeletal muscle is not the only organ whose function limits endurance performance. The heart is a particularly important endurance organ. Endurance training induces the athlete's heart or physiological cardiac hypertrophy. Such a heart has a hypertrophied left ventricle, which increases stroke volume, cardiac output, and the VO_2max (Ellison et al., 2012, Ekblom, 1968). To test whether endurance genes change their expression in a mouse model of physiological cardiac hypertrophy induced through 8 weeks of swimming versus a pathologically hypertrophied heart achieved through isoproterenol treatment versus sedentary controls, I re-analyzed the dataset of Galindo et al (Galindo et al., 2009). This analysis revealed that depending on the measured probe, *Ppargc1a* increased by 4.27 and 6.96-fold, whereas *Thra* decreased -2.50-fold, *Ppard* by -2.86-fold, and *Tnfsf12* by -1.80 fold, in the physiologically hypertrophied heart specifically when compared to sedentary control, respectively (supplementary data S8**).** Some endurance genes alter their expression in response to both physiological and pathological hypertrophy of the heart. *Hif1a* increases by 1.99-fold, *Mef2c* by 1.99 - 2.90-fold, and *Pten* by 2.05-fold during physiological hypertrophy, while *Hif1a* decreases -1.87-fold, *Mef2c* -2.16-fold, and *Pten* by -1.74 -1.84-fold during pathological hypertrophy. *Ppargc1b* and *Hk2* specifically decrease their expression in pathological hypertrophy by -2.80-fold, and -1.95-fold, respectively, and not during physiological hypertrophy. These data demonstrate that endurance exercise can induce or repress the expression of some endurance genes in the heart of mice.

3.1.7 Do endurance genes interact with each other? Do endurance genes share common features?

Next, I investigated whether endurance genes are functionally linked and whether these genes are enriched among specific classes of genes such as genes that share a common domain or molecular function. First, I performed a STRING analysis that predicts direct physical and other associations for a group of proteins (supplementary data S9, (Szklarczyk et al., 2017). Figure 3.5 illustrates the results of this analysis. Clusters in this figure are linked to the mitochondrial biogenesis regulator Pgc1a (encoded by *Ppargc1a*), the calcium/calmodulin-stimulated phosphatase calcineurin encoded by *PPP3CA,* and the β2-adrenoceptor encoded by *Adrb2*. This suggests some functional interaction between endurance genes.

Figure 3.6 String analysis for endurance genes.
Lines between proteins indicate evidence of association.

I then performed a functional enrichment analysis to see whether the endurance performance-increasing genes share common features. It has been pointed out that such enrichment analyses may be erroneous if the enrichment is not adjusted to the transcriptome of the tissue of interest (Timmons et al., 2015). However, the endurance effect of our gene list could be due to expression in several tissues and so I am unable to use one tissue-specific background list. Instead, I have conducted a general meta-enrichment analysis using ToppGene. This analysis has revealed potentially relevant enrichments that include the mouse phenotype “abnormal heart rate” (MP:0001629) for 8 of the genes and “abnormal metabolism” (MP:0005266) for 13 of the endurance genes. All significant enrichments are listed in supplementary data S10.

3.1.8 How many endurance genes/proteins are predicted to be secreted?

Finally, ≈3000 proteins are predicted to be secreted from cells. To find out how many endurance genes are predicted to encode secreted proteins, I retrieved the list of secreted genes/proteins from Protein Atlas (https://www.proteinatlas.org/humanproteome/secretome) and compared these genes with the list of endurance genes. I identified three endurance genes, *Pappa* (Pappalysin 1), *Thbs1* (Thrombospondin 1) & *Tnfsf12* (TNF superfamily member 12) that encode proteins that are predicted to be secreted (supplementary data S11). Together, these secreted proteins could play possible roles in inter-organ signaling in relation to acute exercise or adaptation to chronic exercise.

3.1.9 Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00262/full#supplementary-material

Part 2 - A study on the possible role of Hippo gene-mutated on skeletal muscle phenotype

Publication 2

Authors: Yaghoob Nezhad, F. *, Riermeier. A. J., Schönfelder, M., Becker, L., De Angelis, M. H., & Wackerhage, H. **Title**: Skeletal muscle phenotyping of Hippo gene-mutated mice reveals that Lats1 knockout increases the percentage of type 1 muscle fibers. **Journal**: Transgenic Research

DOI: 10.1007/s11248-021-00293-4

3.1.10 Summary

The Hippo pathway is a signaling transduction network that controls organ size. Given the hippo pathway's roles in other organs, it has been hypothesized that this signaling pathway could be involved in the different regulatory mechanisms in skeletal muscle including satellite cells' self-renewal, proliferation, differentiation, and apoptosis of myogenic cells. In the last two decades, some studies investigated the involvement of the Hippo pathway in the regulation of skeletal muscle structure and function. Whilst transgenic mice for many Hippo genes have been generated, the resultant skeletal muscle phenotype was not always characterized. In this study, I phenotyped and compared the muscles of hind limbs of mice where Hippo genes were mutated (*Lats1*$^{-/-}$, *Mst2*$^{-/-}$, *Vgll3*$^{-/-}$, and *Vgll4*$^{+/-}$ mutated mice) with those of wild-type control mice. I found that Lats1 contributes to the regulation of muscle fiber type proportions, and its expression is regulated by physiological and pathological situations in skeletal muscle.

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Contribution

I am the first author of this manuscript. I performed experiments, analyzed data, and wrote together with Henning Wackerhage the first draft. All authors contributed to the final submitted manuscript.

3.1.11 Effect of Hippo genes mutation on the absolute and relative size of hindlimb muscles

The Hippo signal transduction network regulates transcription through Yap/Taz-Tead1-4 in many tissues including skeletal muscle. Whilst transgenic mice for many Hippo genes have been generated, the resultant skeletal muscle phenotype was not always characterized. Here, I aimed to phenotype the hindlimb muscles of Hippo gene-mutated *Lats1*$^{-/-}$, *Mst2*$^{-/-}$, *Vgll3*$^{-/-}$, and *Vgll4*$^{+/-}$ mutated mice.

First, to gain information on whole-body phenotypes of the Hippo transgenic mice that I planned to investigate, I retrieved publicly available IMPC phenotype data in supplemental table S2. Key phenotypes of Hippo gene-mutated mice were a 52% ($P < 1.91\times10^{-06}$) higher fat mass in Tead1$^{+/-}$ female mice, a 33% ($P < 1.64\times10^{-06}$) higher grip strength (Forelimb and hindlimb grip strength normalized against body weight) in Lats1$^{-/-}$ female mice, and 18.6% ($P < 1.69\times10^{-08}$) less lean body mass in *Lats1*$^{-/-}$ male mice (Fig.1a), as well as a 27% ($P< 2.92\times10^{-06}$) lower grip strength (Forelimb grip strength normalized against body weight) in Lats1$^{+/-}$ male mice when compared to sex- and age-matched wild-type mice (Supplemental table. S3)(see appendix). Next, to study the effect of transgenesis on the skeletal muscle phenotype, I phenotyped hindlimb muscles of *Lats1*$^{-/-}$, *Mst2*$^{-/-}$, *Vgll3*$^{-/-}$, and *Vgll4*$^{+/-}$ mice versus sex- and age-matched wild-type mice as controls. *Lats1*$^{-/-}$ muscles were significantly lighter than those of wild-type controls (Fig.3.10 b) but this is explained by a proportionally lower body weight so that relative muscle weight was unchanged (Fig.3.10 c). I could not detect pathological changes such as central nuclei or central cores in any of the mutant mice (Fig.3.10, e; Fig. s1((see appendix)).

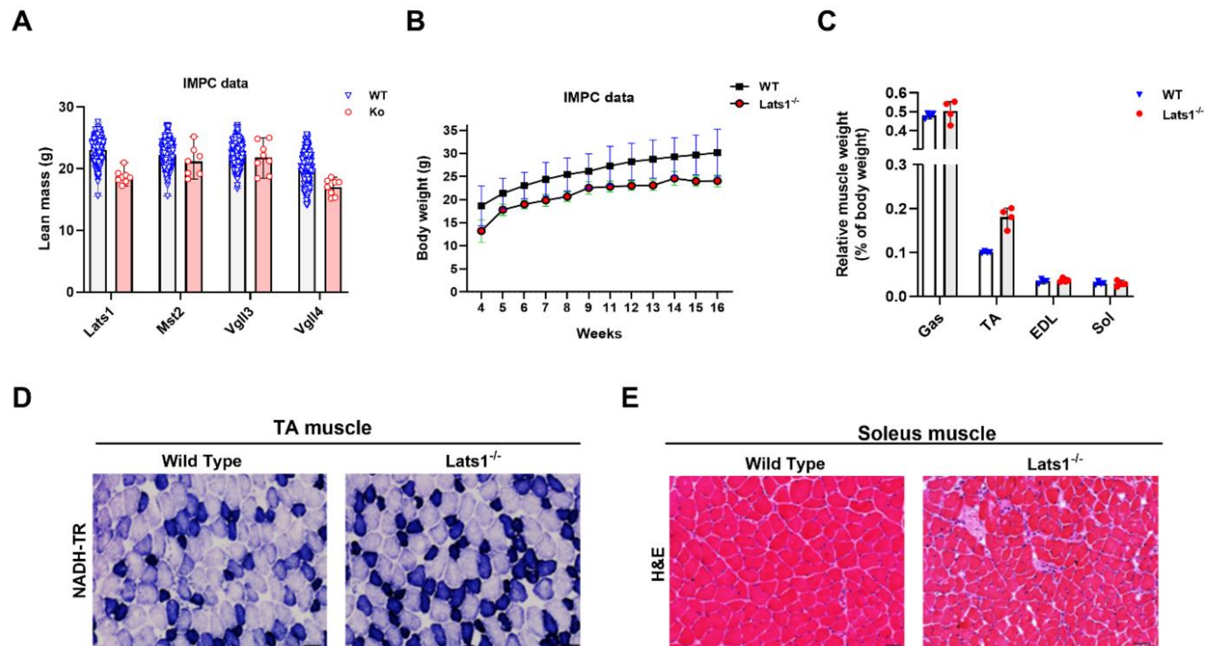

Figure 3.7. Hippo-related genes Knockout mice have normal skeletal muscle structure and function

Average of (a) Lean mass of Hippo related - genes mutated 16-weeks-old, and control mice. (b) Relative muscle weights of Gastrocnemius (Gas), Tibialis Anterior (TA), Extensor Digitorum Longus (EDL), and Soleus(Sol), muscles normalized to total body weight from Lats1$^{-/-}$ mice (n = 4). (c) NADH-Tetrazolium Reductase (NADH-TR) staining of Tibialis anterior muscle cross-sections from control and Lats1$^{-/-}$ mice (high oxidative capacity in dark blue, low oxidative capacity in light blue, and non-oxidative in white) (n = 4). (d) Hematoxylin and Eosin (H&E) staining of the soleus muscle cross-sections from control and Lats1$^{-/-}$ mice (Nuclei in dark blue, cytoplasm in red) (n = 4). Scale Bar = 50 μm. Original data of lean mass, derived from IMPC data. All values present the mean±SEM. *P<0.05. The abbreviation, KO: Knock Out; WT: Wild Type; g: gram; mg: milligram. See Figure Supplementary1(Fig. S.1 (See appendix)) for further H&E and NADH staining.

3.1.12 Lats1 knockout alters muscle fiber type distribution

Next, I quantified the number and size of type I and IIa muscle fibers in the soleus. To do so I differentially stained muscle fibers using an ATPase reaction with an acid (pH = 4.47) pre-incubation which stains type I fibers black and type IIa fibers grey (Figure. 3.11a). This revealed that *Lats1*$^{-/-}$ mice had 11% more type I fibers, and 11% fewer type IIa fibers than age and sex-matched wild-type controls ($p<0.05$; Figure. 3.11b-c). In contrast, the muscles of other Hippo-mutated mice were comparable to their matched wild-type controls (Figure. 3.11b-c and Figure. S.2 (See appendix)). Consistent with this, the concentration of *Myh7* mRNA, which encodes the slow type I myosin heavy chain was 50% higher than in the wild-type mice ($p<0.067$). Consistent with this, the concentration of the type I myosin heavy chain was 80% higher ($p = 0.05$) in *Lats1*$^{-/-}$ mice than in wild-type controls.

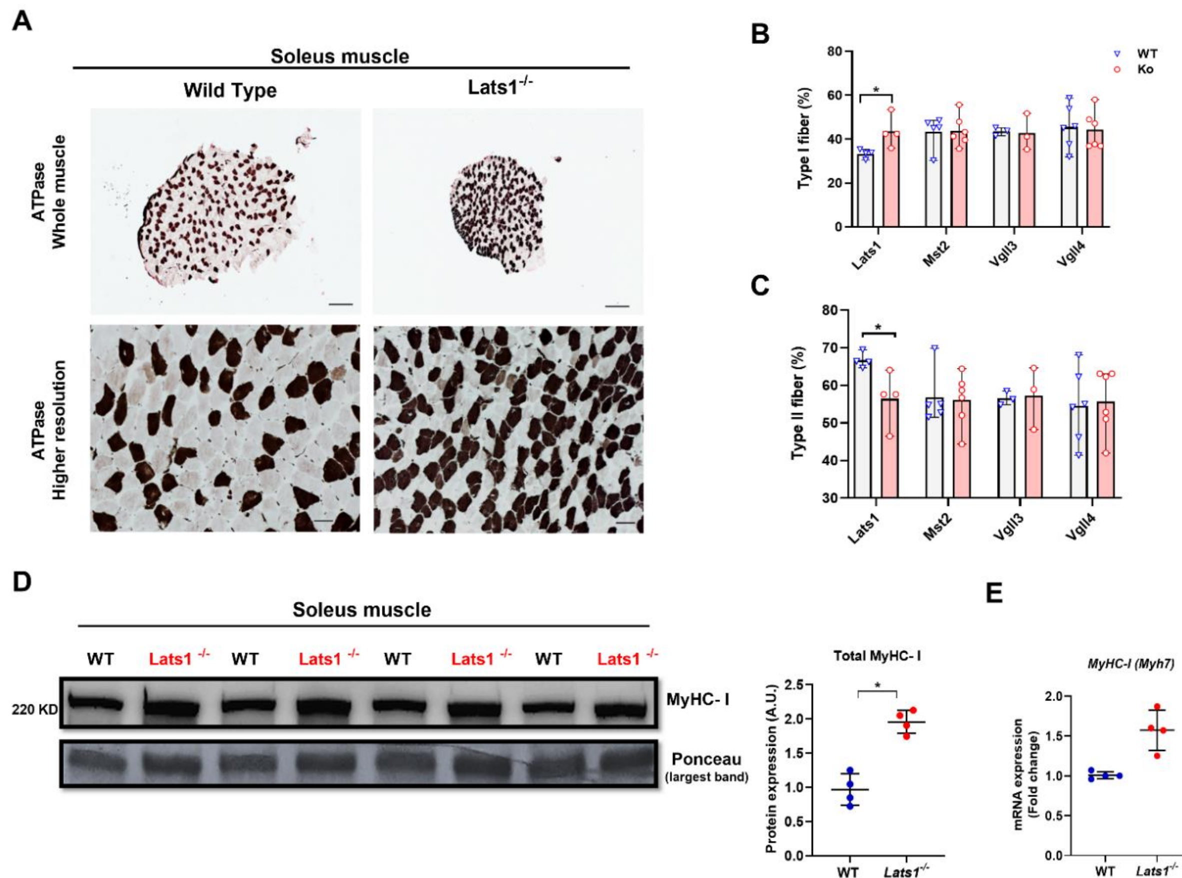

Figure 3.8 Lats1 knockout contributes to muscle fiber type distribution and upregulates slow MyHC expression.

(**a**) Representative images of ATPase stained soleus muscle cross-sections from 16-weeks-old control and *Lats1*$^{-/-}$ mice to determine fiber types (Fibers type 1 stain dark, type 2, and 2a stain light) (n=4). Relative (**b**) fiber type 1 and (**c**) 2a distribution of soleus muscle in control, and Hippo pathway genes mutated mice (n = 3-6). (**d**) Protein expression levels of total MyHC-I in the soleus muscle from control and *Lats1*$^{-/-}$ mice (n = 4). (**e**) Expression levels of the gene encoding MyHC isoform, MyHC-I (*Myh7*) the soleus muscle from control, and *Lats1*$^{-/-}$ mice was measured by qPCR (n = 4). Protein normalized to the largest bond from ponceau staining. *Rpl7* was used as a reference gene to normalize mRNA. The circles indicate individual data points. The abbreviations, A.U, arbitrary units; KD, Kilo Dalton. Scale Bar = 200 µm (whole muscle); 50 µm (higher resolution). All values present mean ±SEM. *P<0.05. See Fig. s2 for further ATPase staining figures.

3.1.13 The expression of *Lats1* and the gene encoding slow(I) myosin heavy chain under the effect of exercise stimuli in-vitro

To investigate whether *Lats1* is regulated by exercise-related stimuli, I incubated C2C12 myotubes with the energy stress-related & AMPK-activating drug AICAR and the hypertrophy-inducing β2-agonist Clenbuterol (Figure. 3.12). I then measured *Lats1* gene expression (Figure. 3.13a-b).

This revealed that after 24 h treated with 100 μM Clenbuterol the expression levels of *Lats1* increased 1.2 fold (Fig. 3.13a) when compared to control. After 24 h of treatment with AICAR (1 mM) mean *Lats1* gene expression increased 1.3 fold when compared to control (Fig. 3.13b).

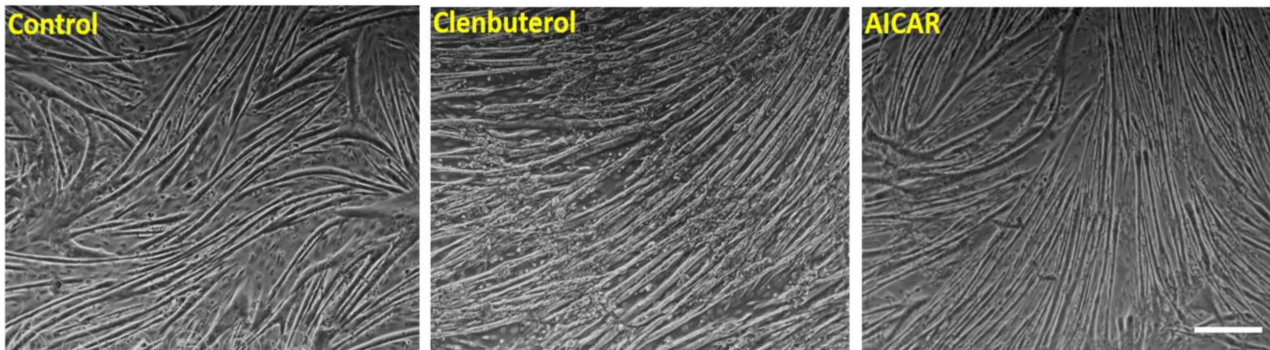

Figure 3.9 The effect of exercise stimuli on C2C12 myotubes.
C2C12 myotubes were untreated (DMSO) or treated with Clenbuterol (100μM), or AICAR(1mM) for 24hrs. Scale Bar= 100μm.

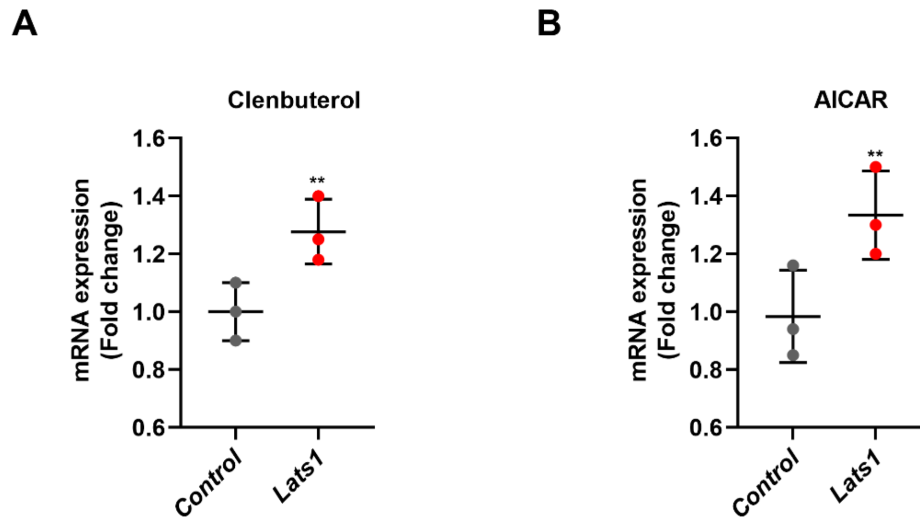

Figure 3.10 The effect of exercise stimuli on the expression level of the *Lats1* gene in vitro. C2C12 myotubes were incubated in (a) Clenbuterol (100 µM), or (b) AICAR (1 mM) for 24 h and analyzed for *Lats1* gene expression by qRT-PCR. *Rpl7* was used as a reference gene to normalize mRNA. The circles indicate individual data points. Data are presented as mean ± SEM., **P<0.001.

3.1.14 Bioinformatical analyses for *Lats1* gene expression in skeletal muscle

To better understand the regulation of *Lats1* expression and phosphorylation in skeletal muscle I retrieved published datasets from Gene Omnibus or downloaded supplemental data from published papers. This revealed that *Lats1* is more expressed in type I muscle fibers in mice than in type II fibers (Chemello et al., 2011). The reanalyzed data from Haslett et al (see Supplementary Data S1A in Haslett et al., 2003) also revealed that mean *LATS1* expression is 63% higher (p=0.014) in the quadriceps of boys with Duchenne muscular dystrophy (DMD) when compared to the normal quadriceps muscle (Haslett et al., 2003a) (Fig. 3.14 a). Moreover, the retrieved microarray dataset GDS4924 data from Lukjanenko et al., 2013 reveal that mean *Lats1* expression increases in mice in regenerating tibialis anterior by 17-77% (Lukjanenko et al., 2013) and in hypertrophying plantaris muscle by 41-71% (See the microarray dataset GSE47098 data from Chaillou et al 2013) (Chaillou et al., 2013) (Fig. 3.14 b-c), suggesting that *Lats1* is transcriptionally regulated in muscle.

In human vastus lateralis, mean *LATS1* expression declines by 19% 2.5h and 21% 5 h (21%) after endurance exercise (Fig. 3.14d) (Vissing and Schjerling, 2014b). However, a MetaMex analysis

(Pillon et al., 2020) revealed no significant expression changes of *LATS1* in different human skeletal muscles after acute or chronic endurance or resistance exercise (Fig. 3.14 e-f).

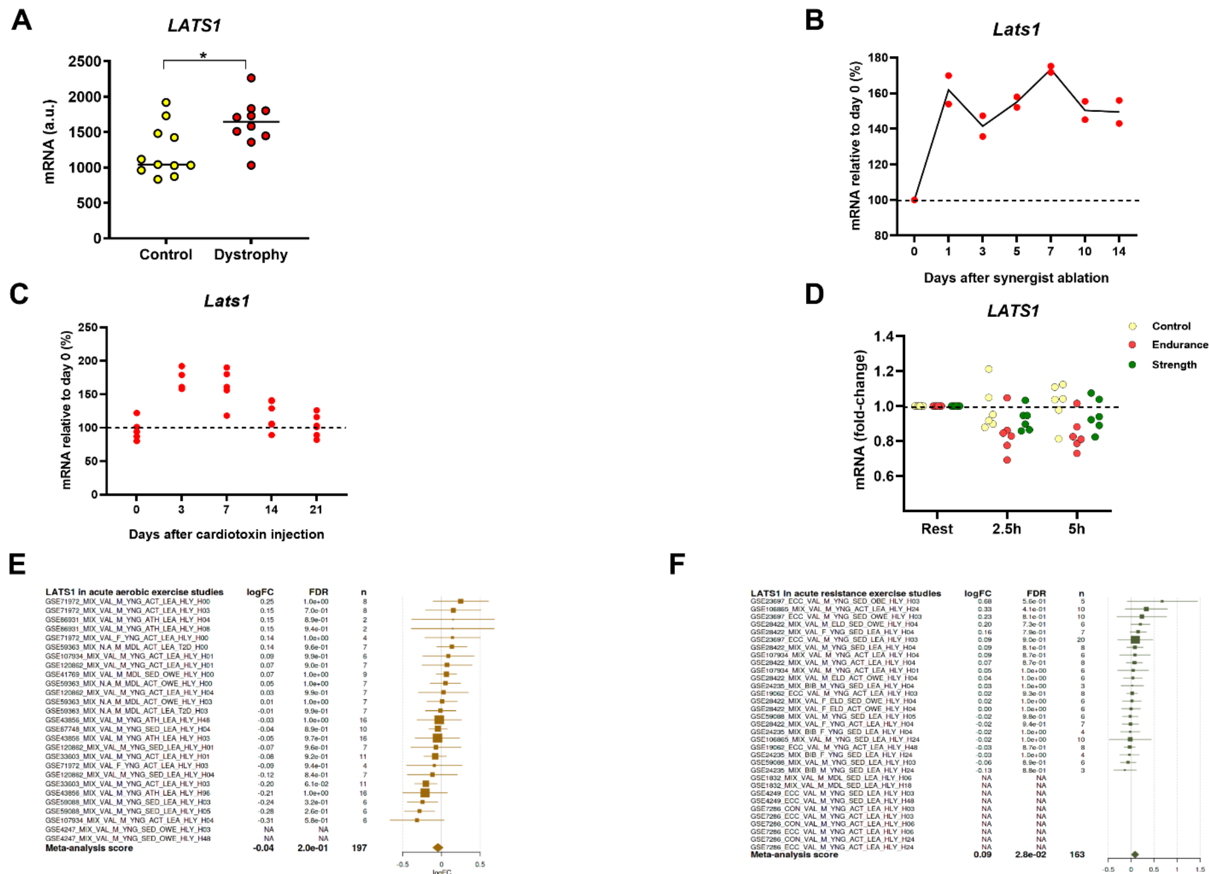

Figure 3.11 *Lats1* gene expression in response to physiological and pathological effects in the skeletal muscle.
(a) LATS1 expression in quadriceps muscle biopsy samples of healthy and DMD patients according to the dataset from Haslett et al (Haslett et al., 2003a). (b) Relative mRNA expression of Lats1 in synergist ablation-overloaded mouse plantaris muscle (Original data derived from microarray dataset GSE47098 data from Chaillou et al 2013). (c) Lats1 gene expression in mice tibialis anterior muscle injured with cardiotoxin injection at day 0 up to 21 (retrieval gene expression data from Lukjanenko et al. 2013). (d) Effect of human endurance and resistance (strength) exercise on the expression of Lats1 gene in the vastus lateralis 2.5 and 5 h after exercise (Vissing and Schjerling, 2014). The circles indicate individual data points. (e-f) A meta-analysis of all available human skeletal muscle studies to obtain a forest plot of the expression of Lats1gene in response to (e) acute aerobic and (f) acute resistance exercises (http://www.metamex.eu/) (Pillon et al., 2020). Muscle Biopsies were collected at 0 hours up to 96 hours after exercise. The fold-change (log2) is represented by a square and the 95% confidence intervals are represented by horizontal lines. The area of each square is proportional to the study's sample size in the meta-analysis. The overall meta-analyzed score is represented by a diamond on the bottom line, the lateral points of which indicate confidence intervals. Abbreviations: logFC, Fold-change (log2); FDR, false discovery rate; n, sample size; A.U, arbitrary units. *P<0.05.

Chapter 4 Discussion

First, I will discuss the results from studies I, and II. Then follows a general discussion with summaries and extensions of the discussions.

4.1.1 Genes whose gain or loss-of-function increases endurance performance

The main finding of this systematic review is the identification of 31 genes whose gain or loss-of-function increases endurance performance in mice by up to 1800% when compared to wild-type control mice. Further bioinformatical analyses reveal the DNA sequence variability of these genes in humans, their organ-specific expression pattern, functional links in-between these genes and the proteins they encode, and a role for some endurance genes during adaptation to endurance exercise. This endurance gene list also provides an up-to-date candidate list for more targeted human genetic analyses for endurance performance or trainability.

4.1.1.1 Relevance of the identified endurance genes for explaining human endurance

A key question is whether the mouse endurance genes are relevant to the genetics of human endurance. Our analyses suggest that this is probably the case. First, human variants of *ACTN3*, *ADRB2*, *BDKRB2*, *HIF1A*, *PPARD*, *PPARGC1A*, *PPARGC1B,* and *PPP3CA* are associated with human endurance (Ahmetov et al., 2016), and human variants of *ADCY5*, *PPARD,* and *HIF1A* are associated with VO_2max trainability (Williams et al., 2017). Second, GWAS studies identified SNPs linked to the human homologs of endurance genes to the hemoglobin concentration (Astle et al., 2016), resting heart rate (Eppinga et al., 2016), metabolic traits (Suhre et al., 2011, Shin et al., 2014), and left ventricular wall thickness (Wild et al., 2017). Finally, I found that the exon DNA sequence of human endurance genes varies considerably. On average, each endurance gene has 174 missense DNA variants, 5 loss-of-function DNA variants, and 11 copy number DNA variants in 60,706 individuals (Lek et al., 2016) note that this data refers to the number of alleles and that the number of carriers is much higher). However, associations or DNA variants of endurance genes do not mean that they actually increase endurance in humans (Houweling et al., 2018). Associations need to be replicated and supported by functional analysis, such as in mouse models, to find mechanisms responsible for endurance phenotypes (Eynon et al., 2017). So far, only for *ACTN3,* there is consistent data showing that a common DNA variant in humans influences muscle

performance, which is similar in the *ACTN3* mouse model (Houweling et al., 2018). Collectively, this suggests that variants of the human homologs of mouse endurance genes could contribute to the variation of endurance-related traits seen in the human population. This has to be replicated in future studies.

4.1.1.2 Several endurance genes affect mitochondrial biogenesis and energy metabolism

The majority of endurance is linked to skeletal muscle metabolism and mitochondrial biogenesis. Here, the transcriptional co-factor Pgc-1α plays a key role (Lin et al., 2002). The authors of the original *Ppargc1a* (which encodes Pgc-1α) overexpression study did not test the endurance capacity of the transgenic mice. However, subsequent studies demonstrated the effect of the overexpression of *Ppargc1a* (Calvo et al., 2008), of the b-isoform of *Ppargc1a* (Tadaishi et al., 2011) and *Ppargc1b* (Arany et al., 2007) on endurance capacity, skeletal muscle mitochondrial biogenesis, and muscle fiber-related gene expression. A related factor is *Ppard* whose overexpression has similar effects on mitochondrial biogenesis, muscle fiber-related gene expression, and endurance capacity (Wang et al., 2004). Many of the other endurance genes regulate the expression of *Ppargc1a* isoforms or of *Ppard* or the activity of the proteins that these genes encode which explains their effect on endurance capacity. *Ppargc1a* expression also increases after endurance exercise in mice (Baar et al., 2002) and human skeletal muscle ((Pilegaard et al., 2003) Figure 2.4) as well as the heart during swimming-induced cardiac hypertrophy (supplementary data S8) suggesting that it is a mediator of muscle and heart adaptations to endurance exercise (Holloszy, 1967).

Two of the endurance genes have been linked to thyroid hormone signaling. They are *Crym*, which encodes a thyroid hormone-binding crystallin (Seko et al., 2016), and *Thra* which encodes a nuclear thyroid hormone receptor (Pessemesse et al., 2012). The mechanisms are probably linked to the effect of thyroid hormones on mitochondrial biogenesis via Pgc-1α and related factors (Weitzel and Alexander Iwen, 2011). Interestingly, some endurance athletes have been reported to take thyroid medication as a treatment (Hart, 2017) which is a concern as the real purpose might be to enhance endurance capacity through thyroid hormone treatment.

Pgc-1α and related factors are, however, not the only regulators of mitochondrial biogenesis, muscle metabolism, and fiber type-specific gene expression. A different group includes *Ppp3ca* which encodes a subunit of the Ca^{2+}-activated phosphatase calcineurin (Jiang et al., 2010), the calcineurin regulator calsarcin-2 encoded by *Myoz1* (Frey et al., 2008), and the calcineurin-

regulated transcription factor Tfeb which promotes mitochondrial biogenesis and other metabolic adaptations (Mansueto et al., 2017). A genome-wide association study has also linked *TFEB* to left ventricular wall thickness and TFEB phosphorylation decreased significantly from pre to post-exercise (Hoffman et al., 2015), suggesting that TFEB may regulate skeletal muscle and heart adaptations to endurance exercise.

4.1.1.3 Some endurance genes have an effect on the oxygen-delivery system

In humans, a key determinant of a high endurance capacity is the VO_2max, which depends on the maximal oxygen transport capacity. This in turn depends on the maximal cardiac output, which is increased in the athlete's heart, and on the oxygen transport capacity of the blood (Bergh et al., 2000, Lundby et al., 2017). Earlier studies reported that cardiac-specific expression of the kinase Mek1 increased cardiac function (Bueno et al., 2000) and that the expression of a dominant negative form of Pi3k in the heart prevented physiological cardiac hypertrophy (i.e. the development of an athlete's heart) after swimming in mice (McMullen et al., 2003). Unfortunately, whether the heart-specific overexpression of these two genes increased exercise capacity in mice was not tested in this study. In another study, researchers overexpressed the catecholamine-related, adenylyl cyclase-encoding genes *Adcy5* and *Adcy8* in the heart. They found that this overexpression increased cardiac contractility and endurance capacity in the transgenic mice when compared to wild-type controls (Esposito et al., 2008). Also, *ADCY5* gene variants are associated with VO_2max trainability in humans (Williams et al., 2017) and for *ADCY5* and *ADCY8* together 659 different missense DNA variants, 17 loss-of-function DNA variants, and 13 DNA copy number variants have been reported for 60,706 humans in the Exact study (Lek et al., 2016). Collectively, this suggests that numerous DNA variants of *ADCY5* and *ADCY8* contribute to the variation of VO_2max trainability and perhaps VO_2max seen in humans. In a different model, the knockout of *Thbs1* (encoding thrombospondin-1) increased skeletal muscle and cardiac capillary density, left ventricular size, and endurance capacity (Malek and Olfert, 2009). Together this demonstrates changed activity of some endurance genes may contribute to the development of an athlete's heart.

Other endurance genes change their expression in the heart in endurance-exercising mice (Figure 2.3; especially *Ppargc1a* appears to increase during physiological cardiac hypertrophy) or are associated with cardiac phenotypes in GWAS studies (Supplementary data S3). Here, the single nucleotide polymorphism (SNP) near *PCK1* was associated with the hemoglobin concentration (Astle et al., 2016). However, it is unclear whether the overexpression of *Pck1* (encoding Pepck) in skeletal muscle can explain an increased hemoglobin concentration (Hakimi et al., 2007).

4.1.1.4 Endurance genes, neural and behavioral mechanisms

Mental fatigue has recently been highlighted as an endurance-influencing factor (Van Cutsem et al., 2017) but we know little about the molecular mechanisms that influence mental fatigue, neural function, and behavior in relation to endurance. Two of the endurance genes are linked to the nervous system. Acetylcholine is synthesized from acetyl-CoA and choline and released from motor endplates to cause muscle fibers to contract. Interestingly, the overexpression of the sodium-choline channel gene *Slc5a7* increased choline transport, endurance capacity but not strength, and physical activity in mice (Holmstrand et al., 2014). The overexpression of *Pck1* in skeletal muscle not only increased the endurance capacity most (Figure 2.1) but made these mice hyperactive in their home cages (Hakimi et al., 2007). How the high expression of a gluconeogenic enzyme in skeletal muscle can increase spontaneous activity in mice is unclear.

A high endurance capacity is important for many sports, is associated with good health, low mortality and many endurance-associated traits are ≈50% inherited. However, how DNA variants contribute to the variation of human endurance and especially of VO_2max and VO_2max trainability is still incompletely understood and some aspects are controversial. The contribution of the "endurance systematic review" study to our understanding of endurance genetics is a list of 31 genes whose gain or loss-of-function increases endurance performance by up to 1800% in mice.

4.1.2 Skeletal muscle phenotyping of Hippo gene-mutated mice reveals that Lats1 knock-out increases the percentage of type 1 muscle fibers

In this study, I compared the hindlimb muscles of $Lats1^{-/-}$, $Mst2^{-/-}$, $Vgll3^{-/-}$, and $Vgll4^{+/-}$ transgenic mice with muscles of sex and age-matched control mice. The major finding of this analysis is that knockout of *Lats1* led to 11% more type I fibers, 50% more Myh7 mRNA, and 80% more type 1 myosin heavy chain in the soleus when compared to wildtype controls. That suggests that Lats1 modulates the proportion of slow type I muscle fibers in mice. Furthermore, *Lats1* gene expression is upregulated in C2C12 myotubes treated with either the hypertrophy-stimulating agent clenbuterol or the energy stress-related drug AICAR. Furthermore, a re-analysis of published datasets revealed that *Lats1* expression can increase after muscle injury and synergist ablation and that *LATS1*

expression is higher in the muscles of young boys with Duchenne muscular dystrophy than in healthy muscles.

4.1.2.1 Lats1 knockout gene contributes to regulating the muscle fiber type distribution in mouse skeletal muscle

The finding that *Lats1* knockout increased the proportion of slow, type 1 fibers fits the earlier discovery that a loss of *Vgll2* in mice reduced type 1 fibers in comparison to wild-type muscles (Honda et al Sci Rep 2017). Given that Lats1 is an inhibitor of Yap/Taz-Tead1-4 and that Vgll2 is a Tead1-4-coactivator, these results seem consistent. This adds the Hippo pathway to the list of pathways that regulate fiber type proportions such as the calcineurin-NFAT, and ERK pathways (Schiaffino and Reggiani, 2011, Ehlers et al., 2014). However, other Hippo gene mutations did not affect the distribution of muscle fiber types and it is unclear why some Hippo genes can have this effect whilst others have not.

4.1.2.2 Regulation of Lats1 expression in the skeletal muscle

Next, I performed C2C12 cell culture experiments and reanalyzed published data sets to find out whether *Lats1* expression or phosphorylation is affected by exercise, damage, disease, or specific drugs. First, I experimentally investigated whether *Lats1* expression is changed in C2C12 myotubes hypertrophy-stimulated with clenbuterol or exposed to energy stress via AICAR. I found that AICAR increases *Lats1* expression by 1.3 fold. Up-regulation of *Lats1* in response to energy stress treatment with AICAR has been reported in different cell models such as in mouse embryonic fibroblasts and human retinal pigment epithelial-1 cells (Philippe et al., 2018). This suggests that *Lats1* expression responds to stimuli that may affect muscle fiber type-specific gene expression. Second, our re-analysis of existing datasets revealed that *Lats1* expression is elevated in synergist ablation-loaded hypertrophying plantaris muscle (Lukjanenko et al., 2013). Additionally, *Lats1* expression is upregulated in regenerating muscles after cardiotoxin-induced injury (Chaillou et al., 2013) (Fig. 4 b-c). The reanalysis of published datasets also revealed that *LATS1* expression is 63% higher in the quadriceps of young boys with Duchenne muscular dystrophy compared to healthy quadriceps muscle (Fig. 4 a). Vita et al 2018 also reported that LATS1/2 kinase activity increases in five different muscles (quadriceps, biceps, diaphragm, gastrocnemius, and EDL) in mdx mice which is a popular model for studying DMD. In DMD muscles, high miR-21 expression leads to an increase in Lats enzyme activity. Activation of both miR-21 and Lats1 can cause direct suppression of Yap activity and results in muscle degeneration and weakness (Vita et al., 2018). These data suggest that Lats1 is transcriptionally regulated in skeletal muscle.

4.2 General Discussion

The main findings of this thesis are that endurance performance is polygenic genotypes and that the Hippo gene *Lats1* contributes to regulating skeletal muscle fiber type distribution in mice. Given that most genes and their functions are highly conserved between mice and humans, this data is also relevant to human sport and exercise-related genotypes. Importantly, transgenic mouse studies demonstrate a causal relation between genetic variants and skeletal muscle phenotypes whereas genome-wide association studies only show that genetic variants and phenotypes are associated. This has also been acknowledged in a recent article of Claude Bouchard, who is the leading pioneer in sport and exercise genetics (Sarzynski and Bouchard, 2020a).

4.2.1 Contribution to the knowledge about the genetics of endurance capacity

A high endurance capacity is one of the most important performance-limiting factors for many sports and it is also associated with good health and low mortality(Morici et al., 2016). However, how genetics contributes to the variation of endurance capacity, the VO_2max, and its trainability in humans is still poorly understood some researchers even question the role of genetics (Lundby et al., 2017). The contribution of the study of "endurance systematic literature review" to our knowledge about the genetics of endurance capacity identifies thirty-one genes whose activation or deletion increases endurance performance up to 1800% in mice. In contrast to GWAS associations, this is a causal relationship.

Variants of six of the genes that we have identified (*ACTN3, HIF1A, ADRB2, PPARD, PPARGC1A, PPARGC1B, BDKRB2,* and *PPP3CA*) are associated with the endurance capacity in humans (Ahmetov et al., 2016) and three genes (*Adcy5*, *Ppard*, and *Hif1a*) are associated with human VO_2max trainability (Williams et al., 2017). GWAS studies also recognize SNPs that are linked to the human homologous of endurance capacity genes to the resting heart rate (Eppinga et al., 2016), left ventricular wall thickness (Wild et al., 2017), hemoglobin concentration (Astle et al., 2016), and metabolic traits (Suhre et al., 2011, Shin et al., 2014).

Many of the endurance genes are linked to biological signaling pathways that are related to endurance, especially skeletal muscle metabolism and mitochondrial biogenesis. Here, the over-expression of the transcriptional co-factor *Ppargc1a* (Calvo et al., 2008), the b-isoform of *Ppargc1a*

(Tadaishi et al., 2011), *Ppargc1b* (Arany et al., 2007), and *Ppard* (Wang et al., 2004) play an important role in skeletal muscle mitochondrial biogenesis, muscle fiber-related gene expression, and endurance capacity. Moreover, *Ppp3ca* (Jiang et al., 2010), *Myoz1* (Frey et al., 2008), and *Tfeb* promote mitochondrial biogenesis (Mansueto et al., 2017). Also, two of the endurance capacity genes including *Crym*, (Seko et al., 2016), and *Thra* (Pessemesse et al., 2012) have been linked to the signaling of thyroid hormone. The mechanisms are likely linked to the impact of thyroid hormones on mitochondrial biogenesis through Pgc-1α and its related factors (Weitzel and Alexander Iwen, 2011).

Lately, mental fatigue as an endurance-influencing factor has been highlighted (Van Cutsem et al., 2017). Two of the endurance capacity genes includes *Pck1* and *Slc5a7* are linked to the nervous system. *Slc5a7* elevated choline transport, and endurance capacity (Holmstrand et al., 2014). *Pck1 gene* in the skeletal muscle not only increased the mice's endurance performance most but elevated also their activity in home cages (Hakimi et al., 2007).

The data generated by exome sequencing 60,706 people includes a huge number of amino acid sequences and/or function-changing DNA variants for these genes (Lek et al., 2016), indicating that there are genetic variabilities in genes that influence endurance and muscle size. Other endurance capacity genes are linked to the oxygen-transporting systems that restrict the VO2max. Here the best VO2max-influencing candidate genes are the *ADCY8* and *ADCY5* genes that enhance cardiac contractility (Esposito et al., 2008). In humans*, ADCY5 gene variants are also linked to VO2max trainability* (Williams et al., 2017) and around 600 types of function-altering DNA variants have been reported for *ADCY8* and *ADCY5* genes (Lek et al., 2016). However, this leaves lots of the identified genetic variability of the VO2max and VO2max trainability unexplained.

4.2.2 Contribution to the knowledge of the role of Hippo proteins in adult muscle

Hippo genes are both regulators of general functions such as proliferation, differentiation, organ size, and metabolism and are expressed in skeletal muscle. For this reason, one research focus is to determine the regulation and function of Hippo genes in skeletal muscle fibers, myoblasts, and

satellite cells (Sun et al., 2017, Figeac et al., 2019, Judson et al., 2013, Feng et al., 2019). By performing the in-vivo experiment on Hippo genes- mutated mice, I found that *Lats1* is expressed in skeletal muscle and alters the percentage of type 1 fibers in the mouse soleus by 11%. Other Hippo-related genes that also affect muscle fiber type percentages are *Taz*, *Vgll2*, *Tead1*, and *Mst1*. Regarding the role of Lats1 in muscle fiber type distribution, I examined the gene expression of Lats1 C2C12 myotubes in response to exercise stimuli and reanalyzed published data sets to obtain a picture of the regulation of Lats1 in skeletal muscle by exercise and other stimuli. In the in-vitro study, I found exercise stimuli (hypertrophy and energy stress-inducing agents) inducing Clenbuterol and AICAR upregulated Lats1 expression. In addition, bioinformatic analysis from previously published data revealed that *LATS1/Lats1* mRNA is significantly elevated in Duchenne dystrophy muscle (Haslett et al., 2003b), increases during regeneration after cardiotoxin injury (Lukjanenko et al., 2013), and is elevated during synergist ablation (Chaillou et al., 2013) in mice skeletal muscle. However, the expression of *LATS1* reduces after 2.5 h and 5 h of endurance exercise in the human vastus lateralis muscle (Vissing and Schjerling, 2014b). These findings suggest an important role for Lats1 in skeletal muscle (in-vivo and in-vitro).

4.2.3 Limitations

Limitations of the endurance capacity-causing genes review are that the manipulated genes are subjectively chosen by the researchers of each study. Moreover, many researchers do not test whether a manipulated gene changes endurance exercise capacity. The consequence is that the list of manipulated genes and the mice that are tested in an endurance test is subjective, resulting in a biased list of endurance genes. Currently, the International Mouse Phenotyping Consortium (IMPC) generates and phenotypically analyses 20,000 mouse lines (http://www.mousephenotype.org/) but the measurement of endurance capacity is not included in the first-line analysis (Brown and Moore, 2012). Here a triage system might be useful so that mice that have increased cardiac function, a higher hematocrit, or other endurance-associated phenotypes like locomotor activity are then also tested in an endurance test.

Another limitation of the study is that the variation of endurance performance also depends on the endurance tests used in the individual studies. For example, if the endurance performance of the same wild-type and transgenic animals is measured in a graded exercise test versus a time trial test then the results will differ. For example, in the *Pck1* mouse study, the researchers found that wild-type mice ran ≈200 m at a speed of 20 m/min whereas *Pck1*-overexpressing mice ran between 2000 m and 6000 m, which is on average ≈1800% more than the wildtype mice. In contrast, when

the same mice were compared in a graded exercise test with an increase of 1 m/min every minute then the *Pck1*-overexpressing mice achieved a maximum speed of ≈50 m/min whereas the wildtype controls ran up to a speed of ≈20 m/min. This is a much smaller increase of 150% (Hakimi et al., 2007). Together this demonstrates that the type of endurance performance test can greatly affect the outcome and differences in percent between wildtype and transgenic mouse strains. Generally, researchers should aim for standardized protocols for running tests to exhaustion or for graded exercise tests. Booth et al give some specific recommendations for such exercise tests in mice (Booth et al., 2010).

A final limitation of this study is that we do not include mouse models where the gain or loss-of-function of a gene reduces endurance performance. Such mouse models can provide important insights into genes that influence endurance performance (Garton et al., 2016). However, the major problem with such mouse models is that it is difficult to judge whether the decrease in performance is because of the reduction of a true endurance-increasing variable or that endurance is decreased because of a disease. For example, we would expect that almost all tumor-bearing mice have a reduced endurance capacity even though the effect of the genetic manipulation causes tumors and does not directly affect endurance-influencing variables.

In the last study, I conducted in-vivo and in-vitro studies to investigate the role of the Hippo gene-mutated in skeletal muscle. The strength of this approach is that we provided the first in-vivo evidence of *Lats1* function in muscle fiber-type distribution. However, limitations are the sample sizes of four *Lats1*$^{-/-}$ versus four size and age-matched wild-type mice. Furthermore, to perform in-vitro experiments in isolated muscle cells of Hippo gene-mutated mice we needed the freshly isolated muscle cells that unfortunately were not available. Thus, we decided to further study the gene expression pattern of Lats1 under the effect of damage, disease, or specific drug conditions which allowed us to have a big picture of the role of lats1 in skeletal muscle.

4.2.4 Future directions

Further studies are still to fully understand the role of Hippo-related genes such as *Lats1* in skeletal muscle structure and function. Hence, a Lats1 overexpression in-vivo (mouse model) and in-vitro (C2C12 myoblasts and Myotubes model) would be useful given that only Lats1 knockout mice have been studied in skeletal muscle. Also, dual Lats1/2 gain and loss of function in the mice model will be informative about their physiological and pathological functions in skeletal muscle. Furthermore,

it also would be interesting to do some endurance exercise test in Hippo genes mutated mice that allows us to study whether these genes affect endurance performance capacity.

Moreover, the genetics of human skeletal muscle and endurance are still poorly understood. This thesis contains over thirty genes whose mutations cause improving endurance to vary in mice. Given their high conservation between men and mice, it seems likely that DNA variants or even epigenetic modifications of these genes contribute to some sport and exercise-related genotypes in humans.

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Thesis Appendix

Table S1 Genes whose transgenesis in mice increases endurance capacity

Gene symbol	Protein name	Transgenesis	Output measure	Increase (%)	Reference
Pck1	phosphoenolpyruvate carboxykinase 1, cytosolic	Conditional overexpression	Time to exhaustion Distance	+ 68% +1800%*	Hakimi et al., 2007
Scd1	stearoyl-Coenzyme A desaturase 1	Conditional overexpression	Time to exhaustion (run) Time to exhaustion (swim) Distance	+989% +35% +170%	Rogowski et al., 2013
Trib3	tribbles pseudokinase 3	Conditional overexpression	Total work	+138%	An et al., 2013
Ppargc1a	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	Conditional overexpression	Time to exhaustion #1 Distance Max speed	+38-44.9% +50-120%* +24-34%	Calvo et al., 2008
Il15	interleukin 15	Conditional overexpression	Time to exhaustion	+110%*	LeBris et al., 2012

Nr4a3	nuclear receptor subfamily 4 group A member 3	Conditional overexpression	Time to exhaustion Distance	+85%* +100%*	Pearen et al., 2012
Ppargc1a (b isoform)	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	Conditional overexpression	Time to exhaustion Distance Max speed	+36-40% +83-100% +35-40%	Tadaishi et al., 2011
Thra	thyroid hormone receptor alpha (mitochondrial T3 receptor)	Global knock out	Time to exhaustion Distance	+65* +95%*	Pessemesse et al., 2012
Mef2c	myocyte enhancer factor 2C	Gain-of-fuction	Time to exhaustion Distance	+75% +94%	Potthoff et al., 2007
Ppard	peroxisome proliferator activated receptor delta	Conditional overexpression	Time to exhaustion Distance	+67% +92%	Wang et al., 2004
Bdkrb2	bradykinin receptor, beta 2	Global knock out	Time to exhaustion	+90%*	Reis et al., 2015
Thbs1	thrombospondin 1	Global knock out	Time to exhaustion Distance Max speed	+67% +80% +11%	Malek & Olfert, 2009
Cd47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	Global knock out	Time to exhaustion	+80%*	Frazier et al., 2011

Myoz1	myozenin 1	Global knock out	Time to exhaustion Distance	+55%* +75%*	Frey et al., 2008
Trpv1	transient receptor potential cation channel, subfamily V, member 1	Global overexpression	Distance	+80%*	Luo et al., 2012
Slc5a7	solute carrier family 5 (choline transporter), member 7	Global overexpression	Time to exhaustion Max speed	+ 65% + 10%*	Holmstrand et al., 2013
Adrb2	adrenoceptor beta 2	Global knock out	Distance	+65%*	Voltarelli et al., 2012
Adcy8	adenylate cyclase 8	Conditional overexpression	Distance	+50%*	Esposito et al., 2008
Tnfsf12	tumor necrosis factor (ligand) superfamily, member 12	Global knock out	Time to exhaustion Distance	+27% +47%	Sato et al., 2013
Ppargc1b	peroxisome proliferative activated receptor, gamma, coactivator 1 beta	Conditional overexpression	Time to exhaustion Distance	+25% +45%	Arany et al., 2007
Tfeb	transcription factor EB	Inducible conditional overexpression	Distance	+ 40%*	Mansueto et al., 2017

Crym	crystallin, mu	Global knock out	Time to exhaustion	+35%*	Seko et al., 2016
Pknox1	Pbx/knotted 1 homeobox	Conditional knock out	Distance	+35%	Kanzleiter et al., 2014
Actn3	actinin alpha 3	Global knock out	Distance	+33%	MacArthur et al., 2007
Ppp3ca	Calcineurin; protein phosphatase 3, catalytic subunit, alpha isoform	Conditional overexpression	Distance	+33%	Jiang et al., 2010
Pappa	pregnancy-associated plasma protein A; pappalysin 1	Global knock out	Distance (median)	+33%	Conover et al., 2016
Hif1a	hypoxia inducible factor 1 subunit alpha	Conditional knock out	Time to exhaustion (swim) Time to exhaustion (uphill)	+30%* +20%*	Mason et al., 2004
Adcy5	adenylate cyclase 5	Conditional overexpression	Distance	+30%*	Exposito et al., 2008
Hk2	hexokinase 2	Conditional overexpression	Time to exhaustion	+30%*	Fueger et al., 2005

Tfe3	transcription factor to IGHM enhancer 3 (transcription factor E3)	Conditional overexpression	Time to exhaustion Distance	+16% + 27%	Iwasaki et al., 2012
Pten	phosphatase and tensin homolog	Conditional knock out	Time to exhaustion Distance	+10% +15-20%	Yue et al., 2016
Sirt4	sirtuin 4	Global knock out	Time to exhaustion Distance	+20% +20%	Laurent et al., 2013

Table S2 Compilation of publicly available IMPC data

Protein (*Gene*)	Gene mutation	IMPC size & muscle phenotypes	Muscle phenotypes (this study)
Mst1 *(Stk4)*	HOM Del	No size & muscle phenotypes phenotype	Not tested
Mst2 *(Stk3)*	HOM Del	No size & muscle phenotypes phenotype	No Muscle phenotype
Lats1 *(Lats1)*	HOM Del	Higher grip strength in female (33%) Lighter absolute lean body mass in male (18.6%)	Higher fiber type I (11%)
Lats1 *(Lats1)*	HET Del	Reduction of grip strength in male (27%)	Not tested
Tead1 *(Tead1)*	HET Del	Higher total body fat mass in female (51.6%)	Not tested
Tead3 *(Tead3)*	HOM Del	No size & muscle phenotypes phenotype	Not tested
Vgll3 *(Vgll3)*	HOM Del	No size & muscle phenotypes phenotype	No Muscle phenotype
Vgll4 *(Vgll4)*	HET Del	No size & muscle phenotypes phenotype	No Muscle phenotype

Abbreviations: HET Del, Heterozygous Deletion; HOM Del, Homozygous Deletion

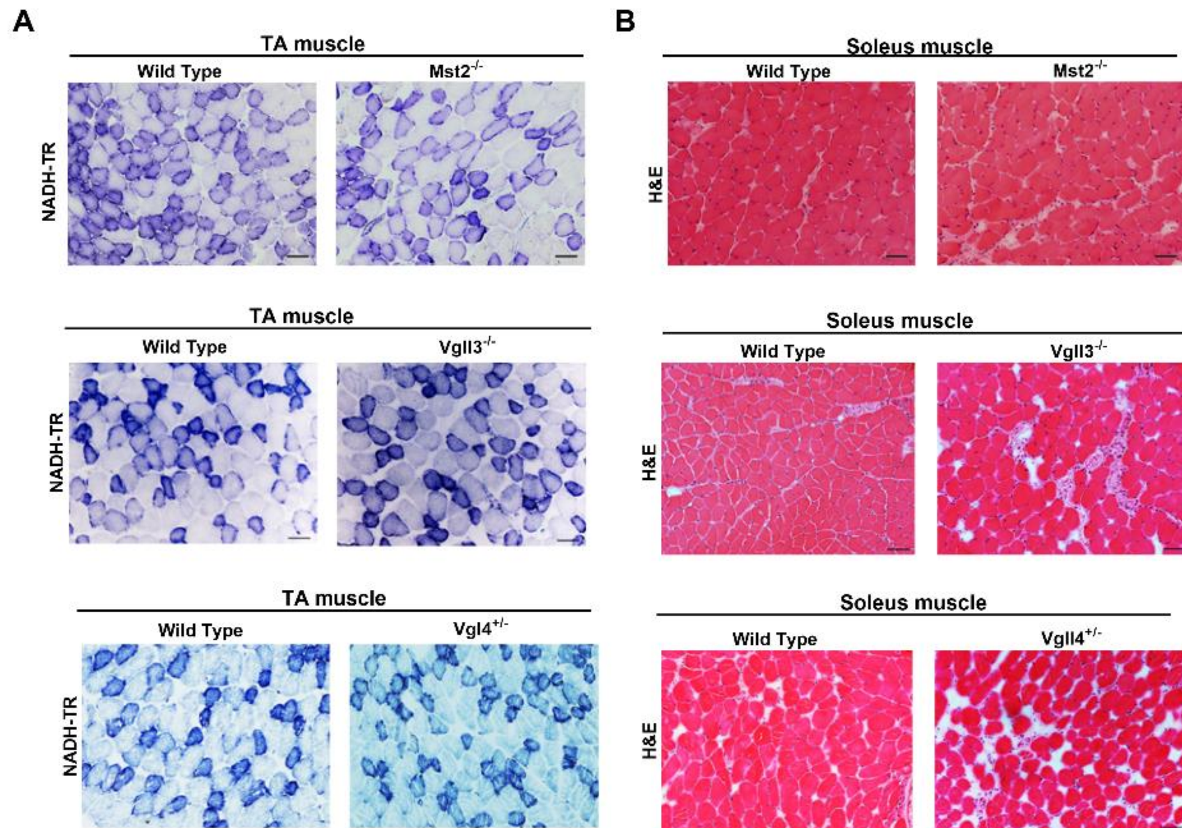

Figure S 1. No Pathological effects in muscle structure and enzyme activity following Knockout of Hippo related genes in-vivo.
(a) NADH-Tetrazolium Reductase (NADH-TR) staining of Tibialis anterior muscle cross-sections from control and Hippo related genes knockout mice (high oxidative capacity in dark blue, low oxidative capacity in light blue, and non-oxidative in white) (n = 3-6). (b) Hematoxylin and Eosin (H&E) staining of the soleus muscle cross-sections from control and Hippo-related genes knockout mice (Nuclei in dark blue, cytoplasm in red) (n = 3-5). Scale Bar = 50 μm.

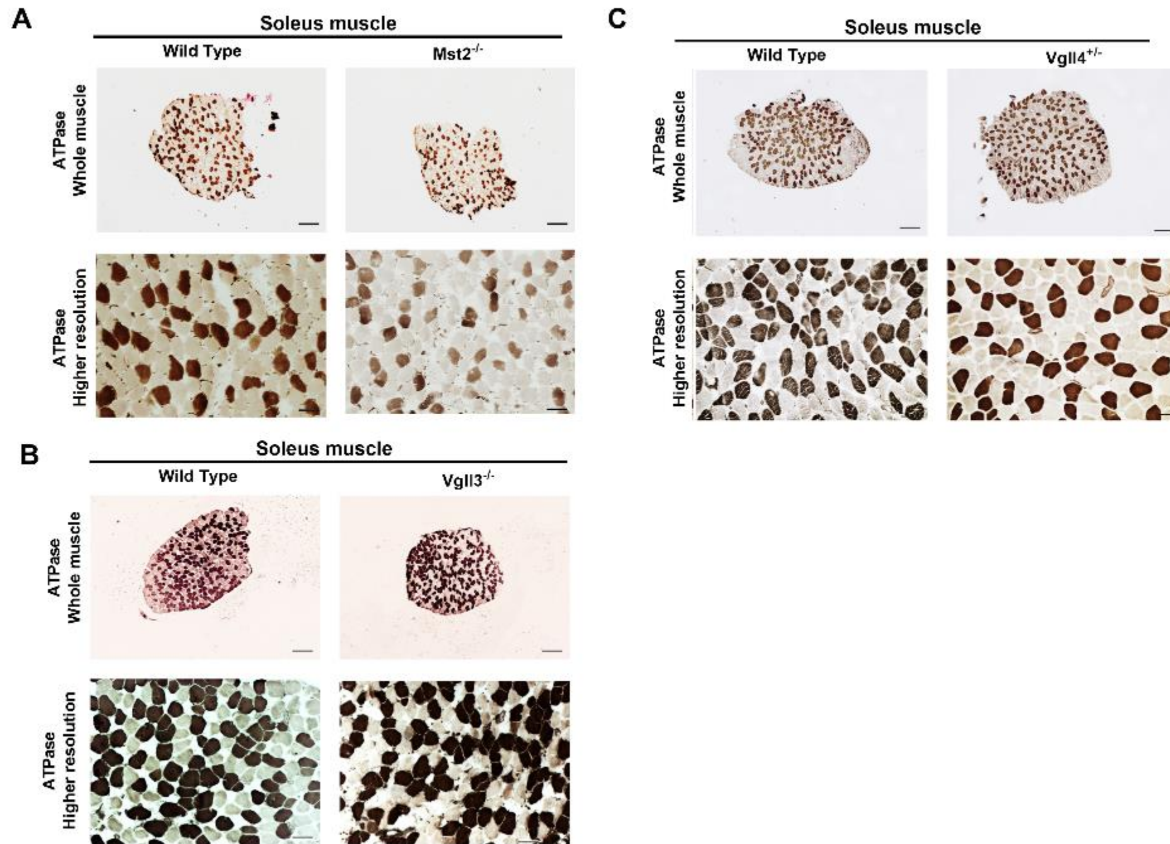

Figure S 2. *Mst2*, *Vgll3*, and *Vgll4* knockout do not affect muscle fiber type distribution and muscle or fiber cross-sectional area (CSA) in Hind limb muscles.
ATPase stained soleus muscle section of (a) wild type or *Mst2*$^{-/-}$, (b) wild type or *Vgll3*$^{-/-}$, and (c) wild type or *Vgll4*$^{+/-}$ mice (Fibers type 1 stain dark, type 2, and 2a stain light) (n = 3-6). Scale Bar= 200 µm (whole muscle); 50 µm (higher resolution).

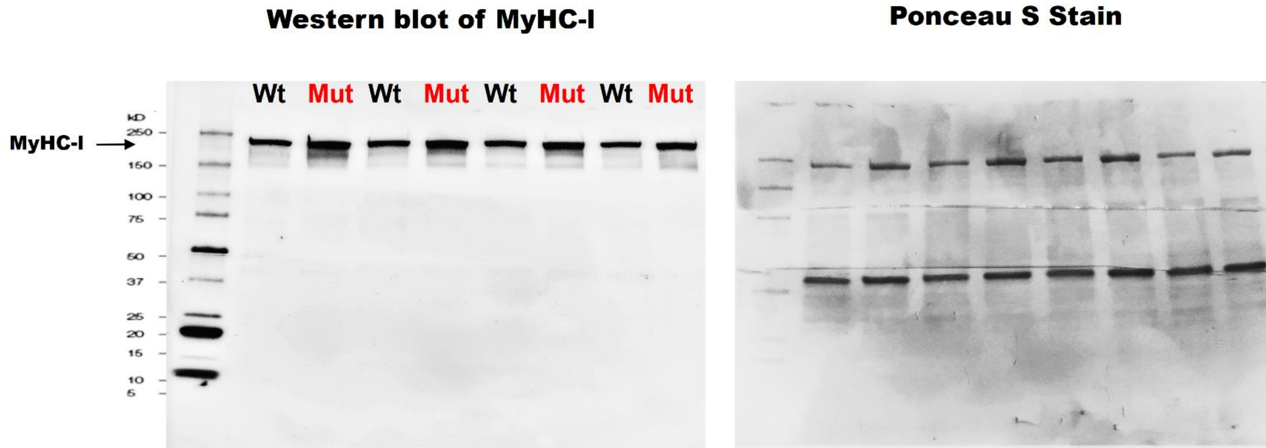

Figure S 3. Full size of western blot membrane from the wild-type and Lats1 mutant mice.

List of publications

First author publication

Yaghoob Nezhad, F. *, Verbrugge, S. A. J., Schönfelder, M., Becker, L., De Angelis, M. H., & Wackerhage, H. (2019). Genes whose gain or loss-of-function increases endurance performance in Mice: A systematic literature review. *Frontiers in Physiology*. https://doi.org/10.3389/fphys.2019.00262

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