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On the interplay between glucose metabolism and skeletal muscle hypertrophy

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II Abstract

Maintaining or increasing skeletal muscle mass is important to prevent age-associated decline in daily functioning. Resistance exercise increases skeletal muscle mass and offers a tool to a prevent muscle loss. Furthermore, resistance exercise increases glucose uptake in skeletal muscle, which is beneficial for glycaemic control. On the other hand, recently it has been proposed that glucose can be used as a building block for anabolic processes, at least in non-muscle cells. We do not know whether glucose metabolism contributes to muscle growth. Therefore, the aim of this thesis is to identify genes and study mechanisms through which skeletal muscle can use glucose for growth processes.

In study I, II and III we searched the scientific literature to identify genes whose gain- or loss-of-function increases skeletal muscle mass in mice, or alter skeletal muscle glucose uptake or increases endurance capacity. We found 47 genes that cause muscle hypertrophy after gene manipulation. Several of these genes are connected to signalling pathways that are able to induce hypertrophy, including 1) IGF1-AKT-MTOR pathway, 2) myostatin-Smad signalling, and 3) the angiotensin-bradykinin signalling pathway. Whether these *hypertrophy genes* also regulate glucose uptake remains to be determined. We found 176 *glucose uptake genes* that alter glucose uptake in skeletal muscle after gene manipulation. 12 of these genes also control muscle mass. 46 of the *glucose uptake genes* respond to acute resistance exercise, which warrant further investigation to determine their role in muscle size regulation.

In study IV, we investigated the contribution of Warburg effect-associated enzyme PKM2 to muscle growth. Resistance exercise in humans for 6 weeks increases PKM2 abundance which is associated with increased muscle fiber size. *In vitro,* we determined that PKM2 is essential for IGF1 mediated myotube growth. Whether glucose incorporation in myotube is controlled by PKM2 remains to be determined.

Together, the work in this thesis shows that candidates involved in glucose metabolism are important for muscle growth. In addition, we found genes previously unrecognised to play a dual role in both glucose metabolism and muscle hypertrophy. Thus, the studies in this thesis suggest that the interplay between skeletal muscle metabolism and hypertrophy warrant further investigation.

III Zusammenfassung

Der Erhalt oder die Zunahme der Skelettmuskelmasse ist wichtig, um altersbedingte Beeinträchtigungen der täglichen Funktionen zu verhindern. Krafttraining erhöht die Skelettmuskelmasse und bietet ein Weg um Muskelverlust zu verhindern. Außerdem erhöht Krafttraining die Glukoseaufnahme in der Skelettmuskulatur, was für die Blutzuckerkontrolle von Vorteil ist. Andererseits wurde vorgeschlagen, dass Glukose als Baustein für anabole Prozesse verwendet werden kann, zumindest in Nicht-Muskelzellen. Wir wissen nicht, ob der Glukosestoffwechsel zum Muskelwachstum beiträgt. Daher ist es das Ziel dieser Arbeit, Gene zu identifizieren und Mechanismen zu untersuchen, durch die der Skelettmuskel Glukose für Wachstumsprozesse nutzen kann.

In den Studien I, II und III untersuchten wir die wissenschaftliche Literatur, um Gene zu identifizieren, deren gain- oder loss-of-function die Skelettmuskelmasse bei Mäusen erhöht, die Glukoseaufnahme der Skelettmuskulatur verändert oder die Ausdauerleistung erhöht. Wir fanden 47 Gene, die nach Genmanipulation eine Muskelhypertrophie verursachen. Mehrere dieser Gene sind mit Signalwegen verbunden, die in der Lage sind, Hypertrophie zu induzieren, darunter 1) IGF1-AKT-MTOR-Signalweg, 2) Myostatin-Smad-Signalweg und 3) der Angiotensin-Bradykinin-Signalweg. Ob diese Hypertrophie-Gene auch die Glukoseaufnahme regulieren, muss noch untersucht werden. Wir fanden 176 Gene für die Glukoseaufnahme, die die Glukoseaufnahme im Skelettmuskel nach Genmanipulation verändern. 12 dieser Gene steuern auch die Muskelmasse. 46 der Gene für die Glukoseaufnahme reagieren auf akutes Krafttraining, was weitere Untersuchungen rechtfertigt, um ihre Rolle bei der Regulation der Muskelgröße zu bestimmen.

In Studie IV untersuchten wir den Beitrag des mit dem Warburg-Effekt assoziierten Enzyms PKM2 zum Muskelwachstum. Krafttraining beim Menschen über 6 Wochen erhöht die PKM2-Häufigkeit, was mit einer erhöhten Muskelfasergröße verbunden ist. In vitro haben wir festgestellt, dass PKM2 essentiell für IGF1-vermitteltes Myotubewachstum ist. Ob der Glukoseeinbau in die Myotube durch PKM2 kontrolliert wird, muss noch untersucht werden.

Zusammengenommen zeigt die Arbeit in dieser Dissertation, dass Kandidaten, die am Glukosestoffwechsel beteiligt sind, wichtig für das Muskelwachstum sind. Darüber hinaus fanden wir Gene, von denen bisher nicht bekannt war, dass sie eine Doppelrolle sowohl im Glukosestoffwechsel als auch in der Muskelhypertrophie spielen. Somit legen die Studien in dieser Arbeit nahe, dass das Zusammenspiel von Skelettmuskelstoffwechsel und Hypertrophie weiter untersucht werden sollte.

IV List of publications

First author publications

Verbrugge, S. A. J., Gehlert, S., Stadhouders, L. E. M., Jacko, D., Aussieker, T., de Wit, G. M. J., … Wackerhage, H. (2020). PKM2 determines myofiber hypertrophy in vitro and increases in response to resistance exercise in human skeletal muscle. *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms21197062

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1 Introduction

Olympians and extreme athletes have pushed the boundaries of what the human body is capable of. Qingquan Ling lifts 170 kg overhead, while only weighing 56 kg[1]. Javier Sotomayor jumped 2.45 m back in 1993[2]. Usain Bolt covers 100 m in just 9.63 s[3]. Sarah Thomas swims 163.8 km in one continuous swim[4]. It is clear that elite athletes depend on an optimal-functioning musculoskeletal system to accomplish these amazing feats. But skeletal muscle is also essential for basic functioning such as breathing, walking and standing upright. For example, a loss of skeletal muscle mass with age impacts daily functioning affecting quality of life (Janssen et al., 2000).

Apart from daily functioning and exercise performance, skeletal muscle plays a critical role in maintaining health and combating disease (Wolfe, 2006). Having sufficient muscle mass and strength is associated with low morbidity and mortality (Gabriel and Zierath, 2017). Muscle is the main reservoir for amino acids, providing blood amino acids to other tissues in times of stress such as starvation (Wolfe, 2006). A loss of muscle mass reduces our chances of survival from illness or severe trauma. Maintaining muscle mass is critical for healthy aging and recovery from chronic disease.

Skeletal muscle has an important function in regulating metabolism and having sufficient muscle mass is important in the prevention of obesity and type 2 diabetes (Wolfe, 2006). Muscle is the largest storage tank for glucose and keeps glycemia levels in check by extracting blood glucose from the circulation (Wasserman, 2009).

Given the importance of skeletal muscle mass for performance and quality of life it is essential to understand how muscle mass can be maintained or increased.

1.1 Skeletal muscle

Most of our body weight is skeletal muscle tissue. On average 38% in men, and 31% in women of total body mass is muscle tissue (Janssen et al., 2000). The amount of muscle mass someone has differs between individuals and depends on the number of muscle fibers and their respective cross sectional area (Lexell et al., 1988).

One of the first systematic studies on skeletal muscle come from Andreas Vesalius. In 1543, he published *De fabrica* with illustrations describing the muscular system with detailed information on each muscle (Silverman, 1991; **Figure 1a,b**). Skeletal muscle attaches to the bone via tendons that direct force to the skeleton. Bundles of individual myofibers make up a

[1] https://www.iwf.net/results/olympic-records/
[2] https://www.worldathletics.org/records/by-discipline/jumps/high-jump/outdoor/men
[3] https://www.olympic.org/athletics/100m-men
[4] https://longswims.com/longest-swims/

muscle. Myofibers are elongated multinucleated cells covered by a basal lamina (Tajbakhsh, 2009). Most of the myofiber is filled with sarcomeres, the basic units responsible for muscle contraction.
Frequently branching sarcomeres form a myofibrillar networks that transmits force across the length and width of the fiber (Willingham et al., 2020). The two most abundant proteins constituting a sarcomere are actin an myosin. More than 50% of muscle protein mass contributes to the contractile machinery of which 18% are myosins, making them the most abundant proteins in skeletal muscle (Deshmukh et al., 2015).

Skeletal muscle is a heterogenous collection of individual fibers with different intrinsic properties. These properties are largely determined by myosin isoforms (Schiaffino and Reggiani, 1996). In adult human muscle three different fiber types can be distinguished: type I, type IIa and type IIx fibers. Type I and type IIa muscle fiber specification is determined before birth, whereas type IIx is expressed after birth. Myosin isoforms are assumed to be the major determinants of maximum shortening velocity and ATPase activity (Schiaffino and Reggiani, 1996). In **Table 1** the characteristics of different fiber types are summarised. The relative proportion of these distinct myosin isoforms determines the contractile property of a muscle.

Table 1. Characteristics of different fiber types in skeletal muscle

	Type I	**Type IIa**	**Type IIx**
Gene	*Myh7*	*Myh2*	*Myh1*
Shortening velocity	Slow	Intermediate	Fast
Force generation	Low	Intermediate	High
Fatigability	Low	Intermediate	High
Mitochondrial density	High	Intermediate	Low
ATPase activity	High (oxidative phosphorylation)	Intermediate	Low (glycolysis)

Skeletal muscle consists not only of myofibers but also motor neurons, capillaries, extracellular matrix and other mononucleated cell populations that are important for skeletal muscle function (Tajbakhsh, 2009). Motor neurons innervate muscle fibers to transfer action potentials initiate muscle contractions (Spudich, 2001). Capillaries branch from arteries delivering oxygenated blood to support energy production. Furthermore, diverse mononucleated cell populations have been identified in skeletal muscle including endothelial cells, B cells, T cells, neutrophils, macrophages, fibroblasts, glial cells and muscle stem cells (Giordani et al., 2019; **Figure 1d,e**). Of these mononucleated cells, the muscle stem cells received most attention. Muscle stem cells, initially described as satellite cells, reside between the plasma membrane of the fiber and the basal lamina (Mauro, 1961; **Figure 1d**). In adult mouse muscle around 7.7% of mononucleated cells are muscle stem cells (Giordani et al.,

2019; **Figure 1e**). In adult muscle, these stem cells are largely inactive unless they need to replace damaged muscle cells (Schultz et al., 1978). During embryogenesis these stem cells are responsible for the development of muscle fibers (Zammit, 2017).

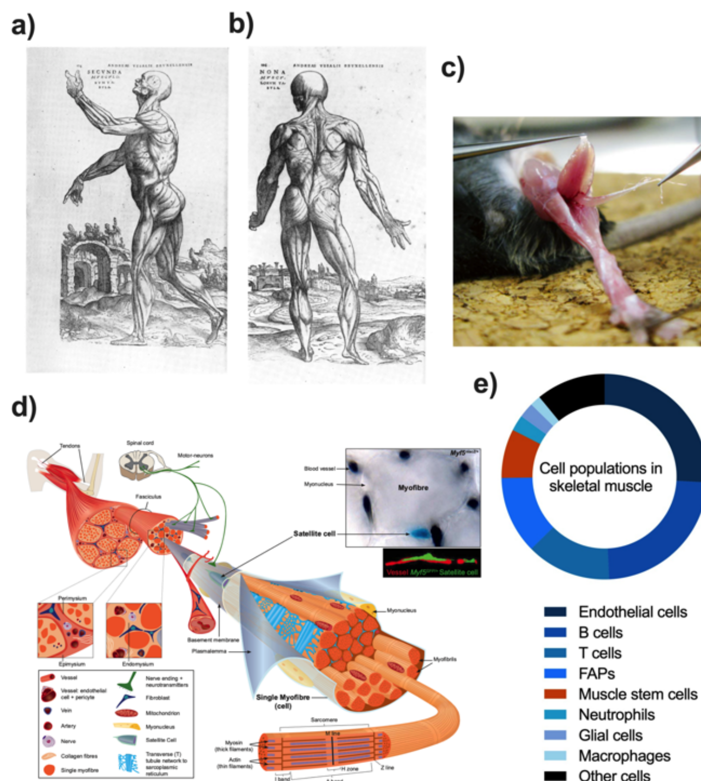


Figure 1. Skeletal muscle structure. (a,b) Muscle men drawn by Vesalius (Vesalius, 1543). (c) Mouse hindlimb musculature (Moyle and Zammit, 2014). (d) Structure of skeletal muscle from gross to microscopic morphology (Tajbakhsh, 2009). (e) Cell populations in skeletal muscle, drawn from (Giordani et al., 2019).

1.2 Skeletal muscle development

During the first days of embryogenesis three germ layers: the ectoderm, mesoderm and endoderm are formed (Sadler, 2019). Skeletal muscle arises from clusters of epithelial cells, termed somites. The formation of these transient embryonic structures can be said to the start of muscle development. In humans this happens in week 3-5 of embryonic development (Musumeci et al., 2015). Somites are clusters of pluripotent epithelial cells and can differentiate into three compartments: sclerotome, myotome and dermomyotome. The fate of somitic cells are specified by extrinsic signals, including Wnt, BMP and sonic hedgehog signalling, that come from surrounding tissues (Buckingham and Rigby, 2014; Zammit, 2017)). Precursor cells from the myotome eventually form the muscles of the back, ribs and abdomen, whereas the dermomyotome contains precursor cells for skeletal muscle including the limbs (Musumeci et al., 2015). Cells of the sclerotome on the other hand give rise to tendon, joints and vertebrae (Musumeci et al., 2015).

Precursor cells in the dermomyotome acquire myogenic potential once they start expressing Pax3 (Buckingham and Rigby, 2014). The proliferating myoblasts that fuse to form adult myofibers are derived from these Pax3 positive precursors found in the dermomyotome (Chal and Pourquié, 2017). Limb muscles derive from Pax3+ precursors that transition from epithelial to mesenchymal cells and migrate from the dermomyotome to the limb bud, an embryonic structure that forms into the limbs. Soon after dermomyotome formation cells begin to express Myf5, followed by MyoD expression, which are the first signs of cells committed to myogenesis. Also, Myf5 and MyoD are expressed in the Pax3+ cells once they arrive in the limb bud (Zammit, 2017; Chal and Pourquié, 2017; Felsenthal and Zelzer, 2017)). Myf5 and MyoD, together with Mrf4 and myogenin form the myogenic regulatory factor family (MRF) which direct progenitors to establish cells that are committed to the muscle lineage. In response to these transcription factors, the dermomyotome will mature in the myotome. Loss of Myf5, MyoD and Mrf4 results in a failure to form skeletal muscle (Rudnicki et al., 1993). indicating the critical role of MRFs in muscle development.

A subset of Pax3+ myogenic progenitors begin to express Pax7 and differentiate into myocytes that fuse together to form multinucleated muscle fibers. These are the first post-mitotic skeletal muscle cells in the embryo where they are formed in the myotome and express myosin heavy chain, actin and desmin (Chal and Pourquié, 2017; Felsenthal and Zelzer, 2017). In humans, the first myotubes appear during week 5 of embryonic development, and most myotubes are matured to muscle fibers around week 8 (Musumeci et al., 2015).

A subset of the Pax7+ progenitors will also form the pool of adult muscle stem cells, termed satellite cells (Relaix et al., 2005). In adult mouse muscle around 7.7% of mononucleated cells are muscle stem cells (Giordani et al., 2019) which are critical for muscle regeneration (Lepper et al., 2011; Sambasivan et al., 2011).

1.3 Skeletal muscle hypertrophy

Skeletal muscle mass depends on the number of muscle fibers and their respective size. The number of muscle fibers is largely determined at birth or shortly afterwards (Li et al., 2015). In wildtype mice, there is no difference in fiber numbers between day 7 and day 56 post-natally (White et al., 2010), suggesting that this largely depends on nature (DNA variation) rather than nurture (environmental influence). For example, mice with a manipulation in the *Fst* or *Mstn* gene have 66% and 86% more fibers compared to mice without this DNA alteration (Lee and McPherron, 2001; McPherron et al., 1997). Still, in early post-natal phase mice gain most of their muscle mass, indicating that hypertrophy of individual fibers is responsible for growth after birth.

Here, the addition of myonuclei is critical (Cramer et al., 2020). Post-natally the number of myonuclei increases until 3 weeks of age while fiber number remain unchanged (White et

al., 2010). Hereafter, new myonuclei needed for muscle hypertrophy must be supplied from myoblasts generated from muscle stem cells (Zammit, 2017). An increase in muscle mass is preceded by an increase in the number of myonuclei, suggesting a causative effect (Bruusgaard et al., 2010).

Skeletal muscle hypertrophy is a gain in protein content from a sustained increase in protein synthesis (Goldberg et al., 1975) that exceeds protein breakdown. Protein synthesis takes place in the ribosomes and ribosomal biogenesis and translational capacity are upregulated during muscle hypertrophy (Figueiredo and McCarthy, 2019; Pereira et al., 2017). Ribosomes consist mainly of rRNA. Mechanical loading increases transcription of ribosomal DNA which over time increases total rRNA levels and thereby the number of ribosomes and thus ability to build new proteins (Figueiredo and McCarthy, 2019).

IGF1-AKT-mTOR is the main pathway regulating protein synthesis in skeletal muscle (Goodman, 2019). Mice with *Igf1* gain-of-function have 30-50% heavier muscles and AKT activation causes muscle fiber hypertrophy and a 2-fold increase in muscle weight (Bodine et al., 2001; Lai et al., 2004a). mTOR signaling is elevated across several muscle hypertrophy models in mice, including postnatal growth, overload-induced hypertrophy and AKT activation (Pereira et al., 2017). mTOR regulates protein translation and ribosome biogenesis, thereby increasing the capacity for muscle to acquire biomass (Figueiredo and McCarthy, 2019; Goodman, 2019).

Independent of AKT/mTOR, the Hippo pathway has been implicated to play a role in muscle mass regulation (Watt et al., 2018). *Tead2* transcription is commonly downregulated in several hypertrophy models (Pereira et al., 2017). TEAD transcription factors are part of the Hippo pathway and interact with YAP, whose manipulation has been linked to muscle mild hypertrophy in mice (Verbrugge et al., 2018a; Watt et al., 2015a).

Another pathway that regulates muscle mass is myostatin signaling. AKT/mTOR regulates cell size in many different cell types including muscle. In contrast, myostatin signaling specifically regulates cell size in skeletal muscle. A loss-of-function mutation in the *MSTN* gene is associated with hypermuscularity in human and causes extreme muscle growth in mice that have up to 162% heavier muscles (McPherron et al., 1997; Schuelke et al., 2004). The genetically modified animals show evidence for the genetic component in skeletal muscle mass. In the most extreme case, myostatin knock-out mice that in addition overexpress follistatin, have muscle weights that increased by 250–350% compared to wild type mice (Lee, 2007)

Muscle mass is regulated by variations in DNA (our genes) (Schuelke et al., 2004; Verbrugge et al., 2018a) and environmental factors such as mechanical load (Wackerhage et al., 2019). Resistance exercise is the main tool at our disposal that we can use to gain more muscle mass in humans.

1.4 Resistance exercise and skeletal muscle hypertrophy

It has not always been recognised that muscle tissue adapts to it environment (Salmons and Sréter, 1976). The type of stimulation that muscle receives from the nervous system and environment drives specific tissue adaptations. Exercise and physical inactivity, aging, and disease induce acute and chronic changes to skeletal muscle (Frontera and Ochala, 2015). Increasing and maintaining muscle mass is important for health (Wolfe, 2006) and resistance exercise offers a tool that can be applied in humans. Resistance exercise places a mechanical load on skeletal muscle which over time leads to muscle hypertrophy (Rindom and Vissing, 2016). 20-24 weeks of resistance exercise increase muscle size on average by ~ 5%, but large inter-individual differences exist (-11% to 30% change) (Ahtiainen et al., 2016). Although, in mice it has been established that myonuclear addition and ribosomal biosynthesis contribute to muscle growth, it is not clear how critical these processes are in resistance exercise induced hypertrophy in humans (Brook et al., 2019). The rate of growth following resistance exercise is smaller and requires a longer period of time to observe muscle gains compared to synergist ablation in mice (Figueiredo, 2019). Still, a gain in muscle fiber size in response to resistance exercise is associated with more satellite cells and myonuclei (Farup et al., 2014; Kadi and Thornell, 2000; Snijders et al., 2016) and increased RNA/DNA content (Brook et al., 2015; Sieljacks et al., 2019). This indicates that myonuclear accretion and ribosome biosynthesis likely play a role in resistance exercise induced hypertrophy in humans. Although the cellular processes are not completely elucidated, it is clear that gaining muscle mass is the result of a positive protein turnover, primarily driven by regulation of protein synthesis exceeding protein degradation (Atherton and Smith, 2012). Protein synthesis depends on activation of mTOR and is essential for protein synthesis and consequent gains in muscle mass (Rommel et al., 2001). mTORC1 inhibition with rapamycin abolishes muscle protein synthesis after acute resistance exercise in humans (Drummond et al., 2009). mTOR is among the most well-studied proteins in muscle hypertrophy induced by resistance exercise, but many unknown genes are likely orchestrating muscle growth that still remain to be discovered. Resistance exercise alters ~2400 transcripts acutely and ~2000 following a period of training (Pillon et al., 2020). Which transcript contribute to muscle hypertrophy requires further investigation.

1.5 Resistance exercise regulates glucose metabolism

Resistance exercise does not only alter mechanical load but also metabolism. Oxygen consumption remains elevated for several hours post-exercise (Børsheim and Bahr, 2003). Resistance exercise interrupts homeostasis and alters metabolite concentrations following

acute (Berton et al., 2017; Tesch et al., 1986) and prolonged periods of exercise (Fazelzadeh et al., 2016) altering metabolic processes including protein anabolism (Biolo et al., 1995; Miller et al., 2005) and RNA synthesis (Sieljacks et al., 2019). Also glucose metabolism responds to acute bouts of resistance exercise, increasing expression of glycolytic enzymes (Pillon et al., 2020) and intermediates (Tesch et al., 1986). Glucose uptake remains elevated up to 24 h after resistance exercise (Bai et al., 2015; Fathinul and Lau, 2009; Marcus et al., 2013a). Glucose is required for energy production in the form of adenosine triphosphate (ATP) to maintain homeostasis. Still, it is not clear what purpose glucose uptake serves 24 h post-exercise. Research in other cell types suggest that glucose can be used as an anabolic substrate to support growth processes (Lunt and Vander Heiden, 2011).

1.6 Glucose as a substrate for anabolism – Warburg effect

Skeletal muscle hypertrophy is regulated by coordinated gene expression and signaling pathways. The mechanisms involved in muscle hypertrophy via IGF1/AKT/MTOR do not only regulate protein synthesis but also have been shown to control glycolysis and glucose metabolism (Elstrom et al., 2004). Activation of AKT or mTORC1 increases glucose uptake (Elstrom et al., 2004; Düvel et al., 2010)and lactate secretion (Düvel et al., 2010). MTOR also stimulates the glycolytic enzymes hexokinase (Hk), phosphofructokinase (Pfk) and lactate dehydrogenase (Ldha). In turn, glucose metabolism regulates the activity of YAP via phosphofructokinase (deRan et al., 2014; Enzo et al., 2015) indicating that pathways associated with skeletal muscle hypertrophy are coupled to metabolism, at least in cancer cells and mouse embryonic fibroblasts.

Otto Warburg was first to observe the phenomenon that cancer cells take up more glucose and synthesize lactate at a higher rate compared to other cells, even in oxygen-rich environments (Warburg et al., 1927). Transplanted tumors in rats took up 70 mg of glucose on average from 100 ml of blood, whilst the glucose uptake of normal tissue was only 10 mg. In addition, the tumors synthesized lactate, which the healthy tissues did not do (Warburg et al., 1927). This phenomenon has further been observed in other proliferating cells including mouse fibroblasts, human lymphocytes, mouse lymphocytes and rat thymocytes (Brand, 1985; Hume et al., 1978; Munyon and Merchant, 1959; Wang et al., 1976). Glucose is not only taken up by the cell to generate ATP, but also serves as a carbon source for the synthesis of amino acids, nucleotides and lipids (Lunt and Vander Heiden, 2011). This raises the question whether also in skeletal muscle glucose can be used as a building block for growth. Although, amino acids are essential building blocks for protein synthesis in skeletal muscle (Reeds, 2000), a collection of independent studies suggests the presence of a Warburg effect in growing skeletal muscle. C2C12 myotubes stimulated with IGF-1 induces myofiber

hypertrophy (Rommel et al., 2001; Semsarian et al., 1999), which has also been shown to increase glucose uptake (Palmer et al., 1997) and lactate production (Semsarian et al., 1999).

1.7 Warburg effect-associated enzymes

Furthermore, IGF-1 induces the expression of genes associated with the Warburg effect. For example, the enzyme phosphoglycerate dehydrogenase (PHGDH; EC 1.1.1.95) uses glycolytic intermediates to initiate *de novo* serine synthesis (Greenberg and Ichihara, 1957). Although, *PHGDH* is low expressed in unstimulated fully differentiated skeletal muscle (GTEx Consortium, 2015) it is essential for muscle development. However, in embryo heads devote of *Phgdh*, genes important for muscle development as well as protein translation are reduced (Furuya et al., 2008; Sayano et al., 2013). Upon IGF1 exposure, *Phgdh* expression increases in C2C12 myotubes (Adams, 2007) suggesting that PHGDH is important for the growth response. Likewise, Brown et al. (2016) observed increased PHGDH protein abundance after inducing hypertrophy in fully differentiated pig muscle (Brown et al., 2016). Together this indicates a role PHGDH in muscle growth.

Another Warburg-effect associated enzymes is pyruvate kinase muscle isoform 2 (PKM2). Pyruvate kinase catalyses the last step of the glycolysis by converting phoshpoenolpyruvate (PEP) to pyruvate. Pyruvate kinase has four isoforms that are tissue specific, with PKM1 predominantly expressed in skeletal muscle (Luo and Semenza, 2012). PKM2 is expressed in proliferating cells (Mazurek, 2011) including muscle stem cells (Ryall et al., 2015). PKM2 exists as either a low-activity dimer or a high-activity tetramer (Zhang et al., 2019). The dimeric PKM2 translocates to the nucleus and controls the transcription of genes involved in cell proliferation and glycolysis (Yang and Lu, 2013). It is assumed that this supports growth by shunting glycolytic intermediates into alternative pathways for biosynthesis (Christofk et al., 2008; Lunt and Vander Heiden, 2011). IGF1/AKT activation increases muscle mass, but also glycolytic enzyme mRNA expression (Akasaki et al., 2014; Lai et al., 2004). Akt promotes the translocation of the PKM2 dimer to the nucleus and thereby increasing glycolytic flux (Salani et al., 2015). Whether PKM2 is involved in skeletal muscle hypertrophy is not known.

2 Aims

Skeletal muscle hypertrophy is regulated by an interplay between environmental and genetic factors. The research contained in the current thesis were designed to investigate the relationship between skeletal muscle hypertrophy and glucose uptake.

My dissertation has two parts.

Part 1 - Genes that regulate muscle hypertrophy and glucose uptake - systematic reviews

In the first study, I examined which genes have been shown to determine skeletal muscle mass in mice. I performed a systematic review and compiled a list of genes whose gain- or loss-of-function increases muscle mass.

In the second study I examined which genes regulate glucose uptake in skeletal muscle. I conducted a systematic review and compiled a list of genes whose manipulation increases or decreases glucose uptake in mouse muscle.

In the third literature review, we compiled a list of genes whose manipulation increases endurance capacity in mice.

Part 2 - First studies on a possible role for Warburg effect-associated enzymes in skeletal muscle hypertrophy

In the first experimental study, I assessed the response of PKM1 and PKM2 to resistance exercise and we determined their role in myotube hypertrophy *in vitro*

In the second experimental study, I contributed to the manuscript where we investigated whether C2C12 myotubes use glucose for protein synthesis and if PHGDH limits myotube growth.

In the third experimental study, I performed initial experiments *in vitro* with muscle stem cells to characterise PHGDH in skeletal muscle.

3 Methods

3.1 Systematic reviews

3.1.1 Search strategies for systematic reviews

To identify publications that identify genes whose transgenesis results in muscle hypertrophy, altered glucose uptake or improved endurance capacity I caried out three systematic review according to the PRISMA guidelines (Moher et al., 2009) and searched the literature according to the PICO framework (Schardt et al., 2007). I used PubReminer (http://hgserver2.amc.nl/cgi-bin/miner/miner2.cgi) to identify relevant MeSH terms and keywords.

Search strategy to identify muscle hypertrophy genes

((((((((mice) OR mouse) OR “mouse model”) OR mice transgenic)) AND ((((((gene transfer techniques) OR “overexpression”) OR “knockout”) OR mutagenesis) OR retroviridae) OR gene deletion)) AND (((((((((“muscle mass”) OR hypertrophy) OR “muscle weight”) OR “hypermuscular”) OR “muscle growth”) OR “muscle fiber size”) OR “cross sectional area”) OR hyperplasia) OR phenotype))) AND skeletal muscle.”

Search strategy to identify glucose uptake genes

((("mice"[mesh] or "mouse model" or "mice, transgenic"[mesh] or "mice, knockout"[mesh] or "skeletal muscle" or "striated muscle" or "muscle")) and ("glucose uptake" or "glucose clearance" or "glucose metabolism" or "glucose tolerance" or "insulin sensitivity" or "glucose tolerance test"[mesh] or "glucose clamp technique"[mesh] or "glucose transport" or "glucose disposal" or "hyperinsulinemic clamp" or "euglycemic clamp" or "hyperinsulinemic euglycemic clamp" or "oxidative glucose metabolism" or "non-oxidative glucose metabolism") AND muscle AND "glucose uptake" NOT (review)

Search strategy to identify genes that improve endurance capacity

(“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).

Inclusion/exclusion criteria

Abstracts were screen and studies were included that met our eligibility criteria. We included articles from peer-reviewed journals, written in English, but excluded reviews. Other distinct exclusion criteria were applied for the three systematic review and were as follows:

Muscle hypertrophy	Glucose uptake	Endurance capacity
Rat or *in vitro* study	Retracted article	Rat or *in vitro* study
No transgenesis or double mutation or miRNA manipulation	Article in other language than English	No transgenesis, double mutation or long non-coding RNA manipulation
Mice showed disease or pathologies or were older than 12 months	More than one intervention (e.g. double gene manipulation)	Major pathological abnormalities result from the gene manipulation
No effect, no outcome measures, or muscle atrophy	Rat or in vitro study	Mice are older than 24 months
No use of a wildtyp or other control group	No use of wildtype or other control group	No statistically significant effect or no outcome measures
	Mice on ob/ob or db/db background	No wild type mice as controls
	No effect on skeletal muscle glucose uptake	Use of an additional drug treatment or dietary supplement (we included studies where a transgene was induced, e.g., through doxycycline injection)
	^{14}C enrichment or FDG-PET as measure of glucose uptake	
	Not first study reporting effect of particular gene manipulation on glucose uptake	

Abstracts that met the eligibility criteria were read full-text. Often during full-text reading, studies appeared to not meet eligibility criteria and yet were excluded.

Data extraction

From the relevant studies that met the inclusion/exclusion criteria I extracted the following data:

Muscle hypertrophy	Glucose uptake	Endurance capacity
Author	Author	Author
Gene name	Gene name	Gene name
Protein name	Mouse alias	Protein name
Method of transgenesis	Human alias	Method of transgenesis
Output measures (e.g. muscle weight, cross sectional area, fiber numbers)	Protein name	Acclimated to exercise?
Muscles studied	Details of transgenesis	Exercise testing protocols
Outcome values for transgenic and control mice	Global or tissue-specific manipulation	Output measure (distance, time, maximal speed)

Relative difference outcome measures between transgenic and control mice	Gain- or loss-of-function	Outcome values for transgenic and control mice
Age of mic	Sex of experimental animals	Relative difference outcome measures between transgenic and control mice
Mouse strain	Number of tested animals	Age of mice
Additional measurements (experimental findings)	Percentage difference in 2DG uptake between transgenic and control mice in soleus, extensor digitorum longus, tibialis anterior, gastrocnemius, quadriceps, or 'other' muscle	Mouse strain
Remarks	In vivo or in vitro study	Additional measurements (experimental findings)
	Increase or decrease in muscle mass	Remarks
	Remarks	

The difference in muscle size, glucose uptake or endurance capacity between transgenic and control mice was often not quantitatively assessed, but only depicted in bar graphs. In that case, we manually estimated the relative difference between transgenic and control mouse from the bar graph. Note, we adopted the official gene name from Uniprot which could vary from an alias used in the original publication.

3.1.2 Bioinformatical analyses

To obtain more information about the identified hypertrophy, glucose uptake and 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.

Muscle hypertrophy review

Research question	**Data source and extraction**
In what tissues are the hypertrophy genes expressed?	Genotype-Tissue Expression (GTEx; RRID:SCR_001618; (GTEx Consortium, 2015).
Are hypertrophy genes differentially expressed in type I vs type II fibers?	GSE23244 (Chemello et al., 2011) from Gene Omnibus (RRID:SCR_007303) and retrieved the data with GEO2R.

Are proteins encoded by hypertrophy genes secreted in blood?	Secreted proteins from the Human Protein Atlas (https://www.proteinatlas.org/; Uhlen et al., 2015) were compared with hypertrophy genes.
Do hypertrophy genes interact?	STRING database analysis (Szklarczyk et al., 2015; https://string- db.org/; RRID:SCR_005223)
Do hypertrophy genes have common functions?	Enrichment analyses using DAVID (Huang et al., 2009; https://david.ncifcrf.gov/summary.jsp; RRID:SCR_001881). Background list of proteins that are expressed in skeletal muscle were used from (Deshmukh et al., 2015)
Are hypertrophy genes associated with human phenotype?	GWAS catalog (MacArthur et al., 2017; https:// www.ebi.ac.uk/gwas/; RRID:SCR_012745)
Are hypertrophy 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 hypertroph genes regulated by mechanical overload in planataris mouse muscle?	GSE47098 data from (Chaillou et al., 2013)
Are hypertrophy genes phosphorylated or dephosphorylated in response to exercise?	Hoffman et al., 2015 supplementary Table S1
Are hypertrophy genes phosphorylated or dephosphorylated in response to electrically evoked muscle contractions?	Potts et al., 2017 supporting information file tjp12447-sup-0001-Table S1.xlsx

Glucose uptake review

Research question	**Data source and extraction**
Are glucose uptake genes transcriptionally regulated by exercise?	Metamex.eu (Pillon et al., 2020)
Are glucose uptake genes phosphorylated/dephosphorylated in	Hoffman et al., 2015 supplementary Table S1

response to high-intensity cycling in humans?	
Are glucose uptake genes phosphorylated/dephosphorylated in response to treadmill running in mice?	(Nelson et al., 2019)
Are glucose uptake genes phosphorylated/dephosphorylated in response to electrically evoked muscle contractions?	Potts et al., 2017 supporting information file tjp12447-sup-0001-Table S1.xlsx (Nelson et al., 2019)

Functional enrichment of identified *glucose uptake genes* shown in **Figure 4** was performed with ToppFun (Chen et al., 2009).

Endurance review

Research questions	**Data source and extraction**
Do endurance genes overlap with DNA variants that are associated with human endurance?	Comparison with list of human genes where DNA variants associate with endurance (Ahmetov et al., 2016)
Do endurance genes overlap with DNA variants that are associated with VO2max?	Comparison with list of human genes where DNA variants associate with VO_2max 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 hypertrophy 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 hypertrophy genes.

The data I extracted to answer to above research questions, I have collected in spreadsheets or plotted in figures included in this thesis or in the published papers.

3.2 Methods experimental studies

3.2.1 Chemical and reagents

Reagent	Source	Reference
3-(N-Morpholino)propanesulfonic acid	Sigma Aldrich	Cat#M1254
5-ehtynyl-2'-deoxyuridine (EdU)	Invitrogen	Cat#A10044
AlexaFluor 488	Invitrogen	Cat#A10266
AlexaFluor 594	Invitrogen	Cat#A10270
Basic FGF	Peprotech	Cat#AF-450-33
Bovine serum albumin	Sigma Aldrich	Cat#A9418
Chick embryo extract	MP Biomedicals	Cat#092850145
Collagenase	Sigma Aldrich	Cat#SCR103
Criterion XT 4-12% Bis-Tris gels	Biorad	Cat#3450125
DMEM	Gibco	Cat#31966
DMEM Glutamax	Gibco	Cat#10566016
EDTA disodium	Sigma Aldrich	Cat#324503
EdU Click-iT	Invitrogen	Cat#C10337
Fetal bovine serum	Thermofisher	Cat#12657029
Goat serum	Dako	Cat#X0907
Horse serum	Gibco	Cat#26050070
magnesium chloride	Sigma Aldrich	Cat#M8266

MESA Blue qPCR MasterMix Plus	Eurogentec	Cat#05- SY2X-03+WOUB
Milk powder	Sigma Aldrich	Cat#70166
N,N-dimethylformamid	Merck	Cat#PHR1553
Penicillin-streptomycin	Sigma Aldrich	Cat#P0781
Phosphate buffered saline tablets	Thermofisher	Cat#BR0014G
Polyvinylidene difluoride membranse (PVDF)	GE Health	Cat#15269894
potassium ferricyanide	Sigma Aldrich	Cat#702587
potassium ferrocyanide	Sigma Aldrich	Cat#P3289
Proteinase K	Biovendis	Cat#405-010
Sodium Acetate	Sigma Aldrich	Cat#S2889
Super Signal West Dura luminol	Thermofisher	Cat#34076
Taq DNA polymerase	Qiagen	Cat#201205
Triton	Sigma Aldrich	Cat#X100
Trypsin EDTA	Sigma Aldrich	Cat#T40949
Vectashield	Vector Laboratories	Cat#CA94010
X-gal solution	Merck	Cat#71077

Laboratory kits

Kit	**Source**	**Reference**
QIAGEN Multiplex PCR Plus Kit	Qiagen	Cat#206152
Quantitect kit	Qiagen	Cat#205311
RNeasy kit	Qiagen	Cat#74104

Equipment

Equipment	**Provider**
ND-1000 spectrophotometer	Labtech
Mx3000P qPCR system	Agilent
Axiovert 200M inverted microscope	Zeiss
Semi-dry transfer blotter	Biorad

3.2.2. Standard buffers

Tail buffer	TBS-T	Transfer buffer	MOPS buffer
50 mM Tris, pH 8.0	150 mM NaCl	25 M Tris	0.2 M 3-(N-Morpholino) propanesulfonic acid
100 mM EDTA	10 mM Tris-HCl;	192 M glycine	0.05 M Sodium Acetate
100mM NaCl	1% Tween-20	20% ethanol	0.01 M EDTA disodium
1% SDS		0.0375% SDS	

3.2.3 Primers

Target gene	**Primer sequence**
Mouse *Phgdh*	F: TGGTGAACGCTAAGCTACTGG R: CAGGGCCACAGTCAGGAG
Mouse *Psat1*	F: AGAATCTTGTGAGGGAATTGCT R: TTTAAGGGGACAGCACTGAAC
Mouse *Psph*	F: CGTTGCTGCAAAGCTCAATA R: CTGTCGGCTGCATCTCATC
Mouse *Pax7*	F: CCGTGTTTCTCATGGTTGTG R:GAGCACTCGGCTAATCGAAC
Mouse *Myog*	F: CTACAGGCCTTGCTCAGCTC R: AGATTGTGGGCGTCTGTAGG
Mouse *Tbp*	F: ATCCCAAGCGATTTGCTG R: CCTGTGCACACCATTTTTCC
Mouse *Myh1*	F: GTCCAAAGCCAACAGTGAAG R: CTTCTGTTTCCATTCTGCCA

3.2.4 Antibodies

I used the following antibodies in my experiments

Immunocytochemistry

Primary antibodies

Target protein	Host	Company	Catalogue number	Working solution
Myod	Mouse	Dako	M3512	1:50
Myog (F5D)	Mouse	DSHB		1:10
Pax7	Mouse	DSHB		1:10
Phgdh	Rabbit	Thermofisher	PA5-27578	1:250

Secondary antibodies

Target protein	Host	Company	Catalogue number	Working solution
α-mouse	Goat	Life technologies	A11029	1:500
α-rabbit	Goat	Life technologies	A11034	1:500

Western blot

Primary antibodies

Target protein	Host	Company	Catalogue number	Working solution
GAPDH	Rabbit	Cell Signaling	Cat#5174	1:30.000
P-AKT^{Ser473}	Rabbit	Cell Signaling	Cat#4060	1:2500
P-P70S6K$^{Thr421/Ser424}$	Rabbit	Cell Signaling	Cat#9204	1:2500
PFK1	Rabbit	Santa Cruz	Cat#sc-377346	1:2500
PHGDH	Rabbit	Cell Signaling	Cat#13428	1:2500
PKM1	Rabbit	Life technologies	Cat#7067	1:2500
PKM2	Rabbit	Cell Signaling	Cat#4053	1:2500
TUBA1 (Tubulin)	Mouse	DSHB	Cat#12G10	1:8000

Secondary antibodies

Target protein	***Host***	***Company***	***Catalogue number***	***Working solution***
α-mouse	*Goat*	*Cell Signaling*	Cat#7076	*1:10.000*
α-rabbit	*Goat*	*Cell Signaling*	Cat#7074	*1:10.000*

All primary antibodies were dissolved in TBST with 5% bovine serum albumin. Secondary antibodies (HRP-linked goat anti-rabbit and goat anti-mouse were diluted 1:10.000 in TBS-T containing 5% milk powder.

3.3. Primary skeletal muscle cells

Satellite cells and floating fibers were isolated as described in detail in Moyle and Zammit (2014). Isolated myofibres were plated on Matrigel and the satellite cell-derived myoblasts then expanded using DMEM GlutaMAX, with 30% fetal bovine serum, 10% horse serum , 1% chick embryo extract, 10 ng/ml bFGF (PreproTech) and 1% penicillin/streptomycin. For immunostaining, cells were plated at a low density in proliferation medium (DMEM GlutaMAX, 20% fetal bovine serum, 10% horse serum, 1% chick embryo extract and 1% penicillin/streptomycin) for 24 hours. EdU (10 µM) was added in fresh proliferation medium for 2 hours at 37˚C. To induce differentiation, cells were plated at a high density for 24 hours and then switched to differentiation medium (DMEM GlutaMAX supplemented with 2% horse serum and 1% penicillin/streptomycin) for 1-2 days.

Proliferation (96 well plate): 5,000
Differentiation (96 well plate): 20,000

3.4 Generation of heterozygous PHGDH reporter mouse

To generate a heterozygous *Phgdh* reporter mouse, the $Phgdh^{tm1a(KOMP)Wtsi}$ allele was obtained from the KOMP repository (https://www.komp.org) and *in vitro* fertilized with eggs from wild-type C57BL/6N mice **(Figure 2)**. Chimera offspring were crossed with C57BL6/N wild type mice obtained from Taconic to generate the first generation heterozygous $Phgdh^{tm1a(KOMP)Wtsi}$ mice. These mice contain a LacZ-neo cassette that can be targeted to visualize PHGDH.

Figure 2. Schematic of PHGDH-Tm1a heterozygous mice

3.5 Molecular methods

3.5.1 LacZ staining

Part of the allele design for our PHGDH mice is an insertion of the *LacZ* reporter gene. The *LacZ* gene encodes the bacterial enzyme ß-galactosidase whose properties can be used to determine endogenous expression levels of *Phgdh* in various tissues. ß-galactosidase activity can be measured with the widely used histochemical approach involving X-gal (Trifonov et al., 2016; Tuck et al., 2015). In cells that express ß-galactosidase, and thus *Phgdh*, X-gal in combination with iron-containing compounds, produces a blue precipitate (Lojda, 1970) indicating the location of *Phgdh* expression.

We perfused pregnant mice with 4% PFA and isolated the embryos (E12.5) which were subsequently fixated for 20 min in 4% PFA at room temperature. Embryos were then washed 3 times with PBS for 15 min. For whole mount staining, we incubated the embryos overnight at 37°C with X-gal reaction buffer (PBS with 1 mg/ml X-gal dissolved in 4% N,N-dimethylformamid, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM magnesium chloride). The following day embryos were washed and incubated in 50% glycerin at 4°C for 5 hours, and finally stored at 70% glycerin until I took pictures.

3.5.2 DNA isolation

To isolate DNA for genotyping, ear clips were digested in 350 µl tail buffer with 10 µl Proteinase K at 56°C overnight.

3.5.3 Genotyping

The mouse genotypes were determined with PCR using the following primers:

Tm1a-neoF	GGGATCTCATGCTGGAGTTCTTCG
Tm1a-F	ACAGCCATCTTCCTTCCACG
Tm1a-ttR	TCCACATTGTCCACACCTGT

Genotypes were determined by PCR using Qiagen Multiplex PCR Plus Kit according to manufacturer instructions, using the following reaction and thermocycler program:

	Volume (µl)
DNA Probe	3
Q-Solution (fach)	5
PCR-Buffer (fach)	2,5
DNTP mix (mM)	0,5
MgCl2	2
Primer Tm1a-neoF	1
Primer Tm1a-F	1
Primer Tm1a-ttR	1
Taq Polymerase (U/µl)	0,2
H_2O	9,7
End volume	25

step	**temp**	**time**	**cycles**
1	94°C	5 min	1
2	94°C	15 sec	40
3	58°C	30 sec	40
4	72°C	1 min 40 sec	40
5	72°C	5 min	1
6	12°C	∞	1

The primers gave the following PCR products

Forward	**Reverse**	**Product length**	**Genotype**
Tm1a-F	Tm1a-ttR	726 bp	Wildtype
Tm1a-neoF	Tm1a-ttR	982 bp	Tm1a

3.5.4 RNA isolation

RNA was extracted from primary skeletal muscle cells using the RNeasy mini kit. All centrifugation took place on a 5415R centrifuge (Eppendorf), at 10,000 rpm. 350 µl of buffer RLT was added, cells were then homogenized by passing the lysate through a blunt 19-gauge needle. 350 µl of 70% ethanol was added before centrifuging for fifteen seconds. 700 µl of buffer RW1 was added to cells and centrifuged for fifteen seconds. 500 µl of buffer RPE was added and the cells centrifuged for thirty seconds. A further 500 µl of RPE was added and cells centrifuged for two mins. New collection tubes were added to each spin column and cells were centrifuged at maximum speed (13,2000rpm) for one min. 40 µl of RNAse-free water was added and centrifuged for 1 min to provide an eluted RNA pellet. Optical density analysis using a Nanodrop ND-1000 spectrophotometer (Labtech) quantified RNA concentration.

3.5.5 cDNA synthesis

Reverse transcription was carried out using the Quantitect kit. RNA was made up to a 12 µl solution using RNAse-free water. 2 µl of DNA wipe-out buffer was added and samples placed in a water bath for two mins at 42℃. Samples were then placed on ice for two mins. 1 µl of primer mix was added and samples were placed in a heat block for two mins at 70℃, followed by an additional 2 mins on ice. 4 µl of RT buffer and 1 µl reverse transcriptase enzyme was added to each sample and placed in a water bath for 30 mins at 42℃. Samples were placed in a heat block for three mins at 95℃. Finally, the cDNA concentration was measured using the Nanodrop.

3.5.6 Real-time quantitative PCR

Primer sequences were designed using Roche Universal Probe Library software (https://lifescience.roche.com/en_de/articles/Universal-ProbeLibrary-System-Assay-Design.html; Roche) (see 3.2.3), or with NCBI Primer Design. Primers were ordered from Sigma-Aldrich and dissolved in double distilled water (ddH20) water to 100 µM and stored at -20 C.

Real-time quantitative PCR (qPCR) was carried out using the Mx3000P qPCR system (Agilent Technologies) using mesa blue master mix solution 10 µl of master mix solution were added to each well of the qPCR plate.

6µl of double distilled water (ddH_20) and 2 µl of the cDNA were then added. 2 µl of the corresponding primer to a final concentration of 2.5 µM was added (see 3.2.3), bringing the total volume in each well to 20 µl. The qPCR cycle consisted of a 10 min incubation at 95℃ for qPCR enzyme activation, followed by 40 cycles at 95℃ for 30 sec, a qPCR amplification

period of 30 sec at 60℃ and a polymerase extension period of 30 sec at 72℃. Data was obtained using MxPro programme software and normalised against *Tbp*.

3.5.7 Immunocytochemistry

Cells or myofibers were fixed in 4% paraformaldehyde for 15 mins. Samples were then washed three times in PBS (5 minutes each wash) and permeabilised using 0.5% triton-X100/PBS for 15 mins. Cells were blocked for 1 hour in 5% goat serum diluted in PBS. Primary antibodies (diluted to the working concentration in PBS) (see 3.2.4) were added to samples and incubated overnight at 4ºC.

Primary antibodies were removed, samples washed three times and appropriate secondary antibodies diluted to the working concentration in PBS added (see 3.2.4). Samples were covered with aluminium foil to avoid light exposure and left to stand at room temperature for 2 hours. Cells were washed again (3 times) and mounted in Vectashield containing 3 µg/ml of 4', 6-diamidino-2-phenylindole (DAPI) to visualise all nuclei.

All images were visualised through an Axiovert 200M Inverted Microscope (Zeiss) and captured using Plan-Neofluar lense and real-time 6D acquisition software (version 4.8) and processed using ImageJ.

3.5.8 Immunoblotting

Protein lysates from human muscle biopsies were provided by Prof. Dr. Gehlert (German Sports University Cologne). I loaded 12 µg (8 µl) of protein in a pre- cast 26-well Criterion XT Bis-Tris 4-12% gel with MOPS buffer for electrophoresis at 180V for ~70 min. After separation the proteins were equilibrated in transferbuffer and transferred to a polyvinylidene difluoride (PVDF) membrane using a semidry blotting system (34 min, 1.2 mA, 25V). After transfer, the membrane was blocked for 1 hour with 5% milk powder dissolved in TBS- T. Membranes were incubated with primary antibodies overnight at 4 degrees (see 3.2.4). Next day, we washed the membrane with TBS-T (first 15 min, subsequently 30 min) and incubated it with matching secondary antibodies (see 3.2.4) for 1 hour at room temperature under constant movement. After washing for 2 hours, we detected the proteins by enhanced chemo-luminescence assay exposed to X-ray film. Films were scanned and quantified using ImageJ software. Proteins were normalized against α-tubulin.

3.5.9 EdU assay

For the detection of proliferation, 5-ehtynyl-2'-deoxyuridine (EdU) at 10 µM was added in fresh proliferation medium for 2 hrs at 37 °C. The click-iT EdU Alexa Flour kit (Invitrogen. ref:

C10339) was used as per manufacturer's instructions with either the 488 (green) or 594 (red) azide to detect incorporated EdU.

3.5.10 Statistics

Shapiro–Wilk tests were used to test for normal distribution. Data were then analyzed using unpaired t-test, two-way analysis of variance (ANOVA), or repeated measures ANOVA for normally distributed data. If data were not normally distributed, I used the Mann–Whitney U test or Friedman's ANOVA for repeated measures. In the case of a significant ANOVA effect, a Bonferroni test was used to determine significant differences between conditions. Significance was set at $p < 0.05$. Data are presented as mean ± SEM with individual data points. Statistical analyses were performed using Prism 7.0.

4 Results

Part 1 – Genes that regulate skeletal muscle mass and glucose uptake

4.1 Genes whose gain- or loss-of-function increases skeletal muscle mass

This original systematic review was published in Frontiers in Physiology in May 2018 by Sander Verbrugge, Martin Schönfelder, Lore Becker, Fakhreddin Yaghoob Nezhad, Martin Hrabe de Angelis and Henning Wackerhage. In this systematic review, we researched the scientific literature to identify genes whose gain- or loss-of-function increases skeletal muscle mass in mice. We found 47 genes that cause muscle hypertrophy after gene manipulation.

Verbrugge, S. A.J., Schönfelder, M., Becker, L., Yaghoob Nezhad, F., Hrabě de Angelis, M., & Wackerhage, H. (2018). Genes whose gain or loss-of-function increases skeletal muscle mass in mice: a systematic literature review. Frontiers in physiology, 9, 553.

Contribution

I am the first author of this manuscript and conducted the systematic literature research, collected data from publications and performed bioinformatic analyses. Henning Wackerhage conceptialized the idea behind this manuscript. Together with Henning Wackerhage and Lore Becker, I wrote the first draft. Me together with all other authors contributed to the final submitted manuscript.

Abstract

Skeletal muscle mass differs greatly in mice and humans and this is partially inherited. To identify muscle hypertrophy candidate genes we conducted a systematic review to identify genes whose experimental loss or gain-of-function results in significant skeletal muscle hypertrophy in mice. We found 47 genes that meet our search criteria and cause muscle hypertrophy after gene manipulation. They are from high to small effect size: *Ski, Fst, Acvr2b, Akt1, Mstn, Klf10, Rheb, Igf1, Pappa, Ppard, Ikbkb, Fstl3, Atgr1a, Ucn3, Mcu, Junb, Ncor1, Gprasp1, Grb10, Mmp9, Dgkz, Ppargc1a (specifically the Ppargc1a4 isoform), Smad4, Ltbp4, Bmpr1a, Crtc2, Xiap, Dgat1, Thra, Adrb2, Asb15, Cast, Eif2b5, Bdkrb2, Tpt1, Nr3c1, Nr4a1, Gnas, Pld1, Crym, Camkk1, Yap1, Inhba, Tp53inp2, Inhbb, Nol3, Esr1*. Knock out, knock down, overexpression or a higher activity of these genes causes overall muscle hypertrophy as measured by an increased muscle weight or cross sectional area. The mean effect sizes range from 5 to 345% depending on the manipulated gene as well as the muscle size variable and muscle investigated. Bioinformatical analyses reveal that *Asb15, Klf10, Tpt1* are most highly expressed hypertrophy genes in human skeletal muscle when compared to other tissues. Many of the muscle hypertrophy-regulating genes are involved in transcription and

ubiquitination. Especially genes belonging to three signaling pathways are able to induce hypertrophy: (a) Igf1-Akt-mTOR pathway, (b) myostatin-Smad signaling, and (c) the angiotensin-bradykinin signaling pathway. The expression of several muscle hypertrophy-inducing genes and the phosphorylation of their protein products changes after human resistance and high intensity exercise, in maximally stimulated mouse muscle or in overloaded mouse plantaris.

4.2 Genes whose gain- or loss-of-function increases skeletal glucose uptake

The following systematic review is in preparation for submission. I conceptualized the idea, performed literature research, analyzed data and am writing the manuscript. Shimon Kempin and Julia Alhusen have contributed with literature research and data analysis. Maximilian Kleinert contributed with ideas and drafting the manuscript.

Systematic literature review

We screened 5597 abstracts from Pubmed and the Mouse Genomics Informatics database. After removing duplicates and applying exclusion criteria, we read 372 articles full-text of which 172 matched our eligibility criteria. The PRISMA flowchart on search and selection of eligible articles is illustrated in **Figure 3**.

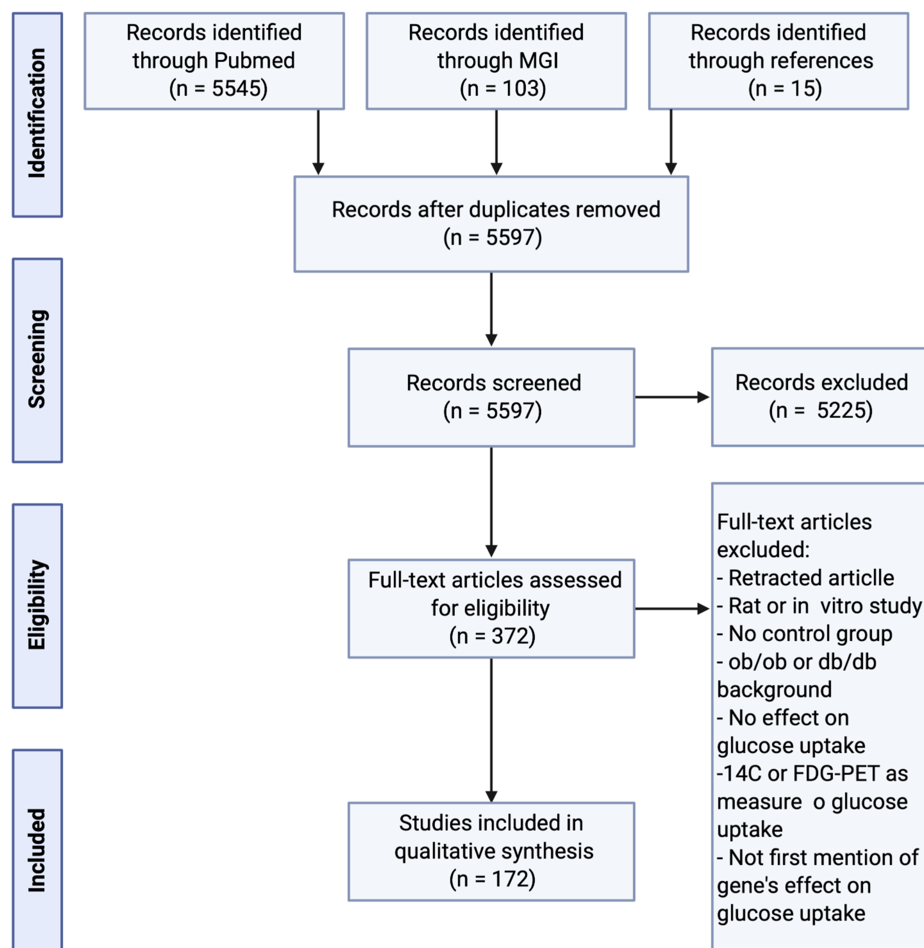


Figure 3. PRISMA flowchart on search and selection of eligible articles.

Which genes regulate skeletal muscle glucose uptake in mice?

From 172 articles we identified 176 genes whose manipulation changed glucose uptake in skeletal muscle **(Table S1-3)**. Gain-of-function of 31 genes increased glucose uptake, while

24 decreased glucose uptake. Loss-of-function of 69 genes increased glucose uptake, whereas for 65 this decreased glucose uptake. From hereon we refer to the 176 identified genes as 'glucose-uptake genes'. In **Figure 4** we plotted the relative changes in glucose uptake between wildtype and transgenic mice for basal, insulin- and contraction-stimulated glucose uptake. Manipulation of genes alters basal glucose uptake from a 330% increase (Slc2a1, also known as Glut1) to a 70% decrease (Sik2) (**Figure 4a, Table S1**). Changes in insulin-stimulated glucose uptake after gene manipulation ranged from +313% (Itga2) to -70% (Hdac3) (**Figure 4a, Table S2**). In addition, we identified several genes that affect skeletal muscle glucose uptake after a muscle contraction protocol or exercise. Here, the effect sizes ranges from a 75% increase (Glp1r) in glucose uptake to a 65% decrease (Chga) (**Figure 4a, Table S3**).

Glucose-uptake genes that are regulated by exercise and inactivity

To identify novel regulators of exercise-responsive glucose uptake, we cross-referenced the 176 *glucose-uptake genes* with recent studies on global gene and signaling changes in response to exercise or inactivity in human and rodent skeletal muscle.

Figure 5 shows *glucose-uptake genes* with changed expression following acute aerobic exercise and acute resistance exercise in human skeletal muscle. Overall, acute exercise altered 63 *glucose-uptake genes*, with distinct changes for aerobic vs resistance-type exercise. 19 *glucose-uptake genes* responded to both types of exercise (**Figure 5a-c**).

Increased sedentary time (e.g. physical inactivity) is associated with a greater risk for type 2 diabetes and the metabolic syndrome (van der Berg et al., 2016), while physical activity reduces this risk for developing T2D (Aune et al., 2015). Bed rest or limb immobilization, both experimental models for inactivity, alters the expression of 34 glucose uptake genes in human skeletal muscle. Of those, 18 increased, while 16 glucose uptake genes were decreased (Pillon et al., 2020) (**Figure 5d**). Of note, exercise reciprocally regulates ~40% of these genes, indicating the importance of exercise to reverse the molecular fingerprint of physical inactivity in skeletal muscles (**Figure 5e**).

Exercise- and contraction-induced protein phosphorylation changes

In addition to transcriptional alterations (Pillon et al., 2020), exercise rapidly and comprehensively changes phosphorylation of proteins. For instance, more than 560 proteins are differentially phosphorylated in human muscle after a vigorous bout of exercise (Hoffman et al., 2015). Exercise in humans alters phosphorylation of 45 phosphosites on 19 unique proteins mapping to the *glucose uptake genes*. 16 of these proteins contained increased phosphorylated sites and 6 proteins decreased phosphorylation **(Figure 6)**. In mice, treadmill running and electrically-stimulated muscle contractions altered 56 phosphorylation sites on 24

proteins transcribed by *glucose uptake genes*. Electrically-evoked muscle contractions in rat alters 63 phosphosites on 22 proteins encoded by *glucose uptake genes* (Nelson et al., 2019).

Taken together exercise and muscle contractions regulate phosphorylation sites on 34 proteins encoded by the glucose-uptake genes proteins, with 27 exhibiting increased and 16 decreased phosphorylation (some proteins have both increased and decreased phosphosites). 17 of these are *glucose uptake genes* are also transcriptionally regulated by either exercise or inactivity **(Figure 5)**. Overall, transcriptomic and phosphoproteomic analyses reveal that acute exercise regulates 79 *glucose uptake genes* in skeletal muscle.

Identifying novel exercise-responsive glucose uptake genes/proteins

To understand which of these *glucose uptake genes* have not been previously associated with exercise, we parsed them through a systematic *Pubmed* search ([(gene name OR aliases OR "protein names" AND exercise)]. As expected, some of our hits are well known to be exercise-responsive: GLUT4, AMPK (PRKAB1, PRKAB2, PRKAA1, PRKAA2), TBC1D1 and TBC1D4 (Sylow et al., 2017). Excitingly, our analysis for the first time connects several glucose-uptake genes to exercise signaling in skeletal muscle. These include DOC2B, STX4 and PEA15, which play a role in in GLUT4 translocation to the plasma membrane (Jaldin-Fincati et al., 2017; Ramalingam et al., 2012; Vigliotta et al., 2004a). TIMP3 and CEACAM1 are regulators of angiogenesis (Menghini et al., 2012; Park et al., 2006). STEAP4, HFE, ABCC9 and RRAD regulate ion metabolism (Chutkow et al., 2001a; Huang et al., 2007; Ohgami et al., 2006). We also identified the transcription factors PKNOX1, ID2 and HMGA1, and the protein kinases SIK1, SPHK1 and DMPK. Collectively, our novel identifications span the regulation of glucose uptake from glucose delivery, glucose transport (GLUT4 translocation) and glucose metabolism.

Glucose uptake and skeletal muscle mass are controlled by common genes

To find out what genes are able to control glucose uptake and skeletal muscle mass, I compared the two above systematic reviews I conducted previously. This revealed that a subset of genes have the ability to both coordinate glucose uptake and regulate muscle mass. *Foxc2, Fst, Ccn5, Cpt1b, Hdac3, Insr* and *Steap4* are positive regulators of glucose uptake and muscle mass **(Table S1-S3)**. Positive regulator is defined as a gene whose gain-of-function increases glucose uptake and muscle mass, or whose loss-of-function decreases glucose uptake and muscle mass. In contrast, negative regulators are genes whose gain-of-function decreases glucose uptake and muscle mass, or whose loss-of-function increases muscle mass. These include *Igfbp3, Tnfsf11, Inpp5k, Mstn, Nos2* **(Table S1-S3)**.

a)

Basal

Slc2a1
Aifm1
Camkk1
Cd36
Ppargc1a
Cbl
Mcat
Vamp8
Crtc3
Mlxip
Lrp1
Trp53
Bmp4
Igfbp1
Mtor
Ppif
Ptpn1
Ppp3cc
Lipa
Abhd5
Ogt
Dgat2
Thbs1
Kcnj11
Pid1
Sgk1
Abhd6
Id2
Hk2
Ddah1
Keap1
Mstn
Ptprv
Rxrg
Pik3r1
Kcna3
Ggps1
Hfe
Cnr2
Foxo3
Rab28
Fabp3
Igfbp3
Lnpep
Ppp1r3c
Gys1
Prkcb
Foxo1
Sparc
Tpm3
Cmklr1
Cebpa
Nos3
Pea15
Nrip1
C5ar2
Chga
Ppp1r3a
Sik2

-100 0 100 200 300

Change in glucose uptake (%)

Insulin-stimulated

Itga2
Pknox1
Pten
Cd44
Trib3
Pik3c3
Slc2a1
Inpp5k
Ilk
Bcl6
Cat
Ucp1
Foxc2
Sik1
Ghsr
Glp1r
Vamp8
Rarres2
Map3k8
Cnr2
Hbegf
Sirt6
Ppp3cc
Fst
Mapk8
Timp3
Slc2a4
Txnip
Slc2a12
Slc2a2
Camk4
Tpm3
Abcc6
Abcc9
Bmpr1a
Kcna3
Cebpb
Ccn5
Mss51
Mlxip
Hk2
Sphk1
Rac1
Pten
Socs3
Pik3r1
Hmga1
Slc13a5
Nos2
Ucn2
Sgk1
Prkca
Gnas
Il10
Tsc1
Cept1
Abhd6
Apob
Myo1c
Tbc1d1
Appl1
Enpp1
Pea15
Ank2
Prkag3
Igf1r
Snca
Lipe
Dgat1
Prkab2
Rictor
Ptpn11
Igfbp1
Vegfa
Rrad
Csrp3
Inppl1
Arntl
Lmna
Lnpep
Ins2
Dmpk
Pparg
Appl2
Ucp3
Dgat2
Esr1
Sirt2
Adm
Tbc1d4
Enho
Irs2
Sgcb
Atp10a
Insr
Rxrg
Ceacam1
Mmp9
Il6
Pikfyve
Igf1
Ankrd26
Nos3
Dlk1
Stxbp3
Itgb1
Sirt3
Doc2b
Col5a3
Cpt1c
Steap4
Sparc
Tnfsf11
Lpl
Stx4a
Sik2
Gfpt1
Ptprf
Prkci
Akt2
Agt
Cyp2c23
Cpt1b
Cav3
Irs1
Nob1
Arfgef3
Hdac3

-100 0 100 200 300

Change in glucose uptake (%)

GOF

LOF

Contraction-stimulated

Glp1r
Mcat
Sod2
Prkcd
Dgat1
Prkaa2
Myo1c
Tlr9
Prkaa1
Rac1
Bcl2
Il6
Tbc1d1
Becn1
Ncf1
Stk11
Chga

-100 0 100 200 300

Change in glucose uptake (%)

b)

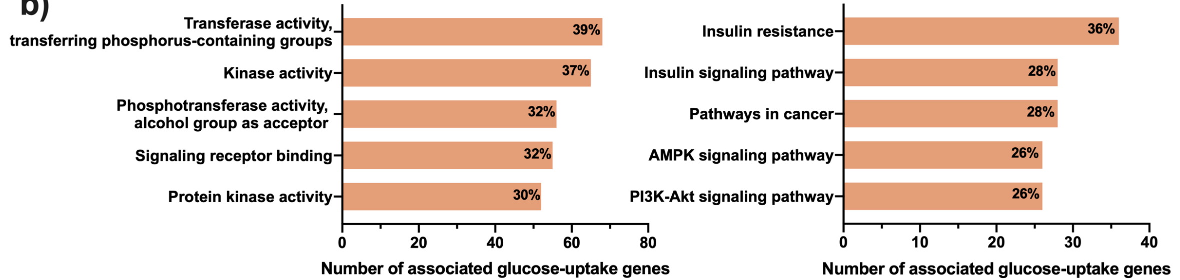
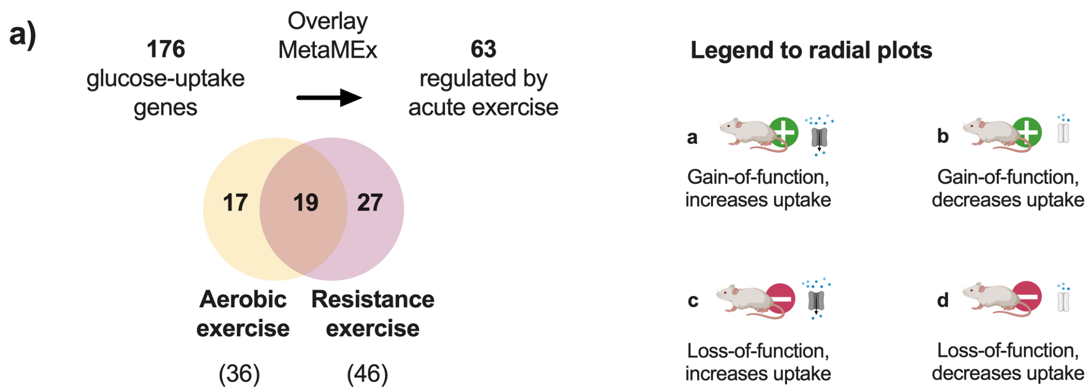
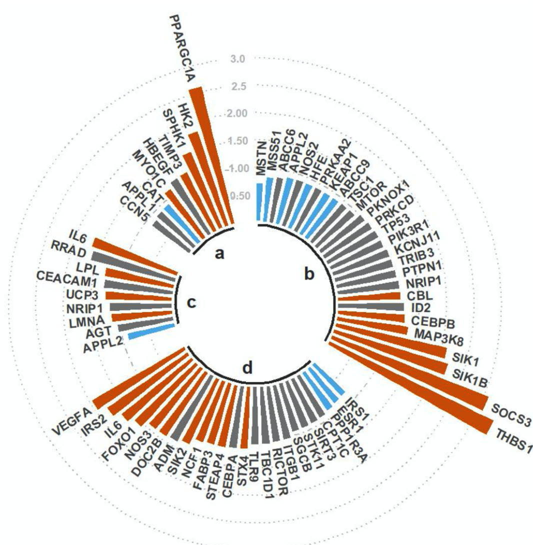


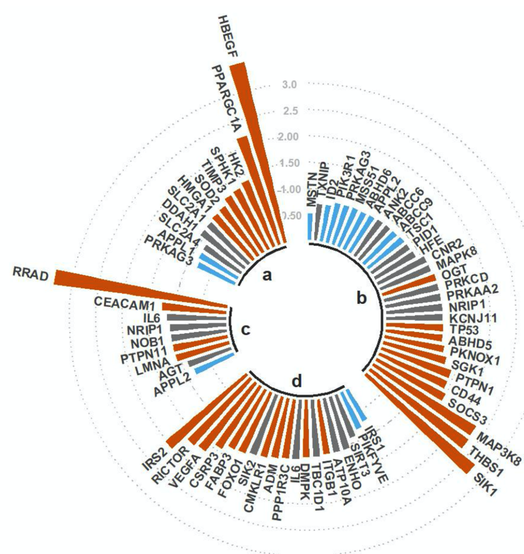
Figure 4. Changes in glucose uptake in mice with gene gain- or loss-of-function. **(a)** Changes in basal, insulin-stimulated, and contraction/exercise-stimulated glucose uptake in transgenic mice compared to controls. GAIN = Gain-of-function (red), LOSS = loss-of-function (blue). **(b)** Functional enrichment for GO molecular functions and KEGG pathways of *glucose uptake genes*. Note, that the manipulation of the same gene can lead to both an increased and decreased glucose uptake depending on the testing conditions (basal vs insulin-stimulated vs contraction-stimulated) (*Cnr2, Rac1, Tbc1d1, Igfbp1, Dgat2* and *Rxrg*).



b) **Glucose-uptake genes in response to acute aerobic exercise**

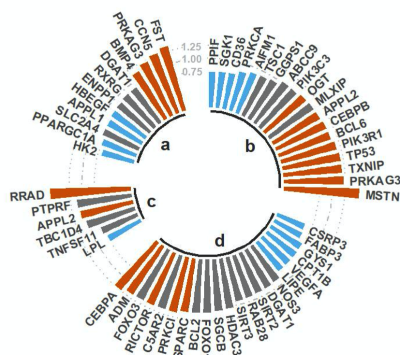


c) **Glucose-uptake genes in response to acute resistance exercise**



d)

Glucose-uptake genes in response to inactivity



e)

Inactivity-induced, but reciprocally regulated by exercise

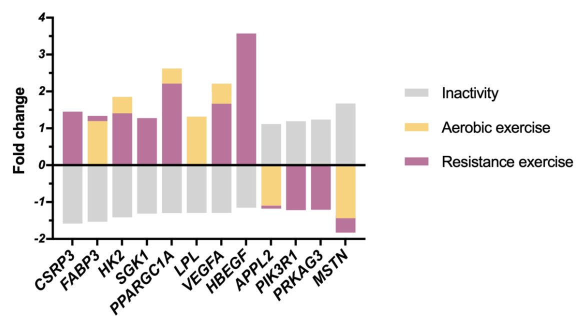


Figure 5. Transcriptional regulation of *glucose uptake genes* in response to acute exercise. **(a)** Overlap analysis with MetaMEx database: 82 of 176 glucose genes respond to acute aerobic

exercise and/or resistance exercise (FDR <0.01) **(b)** mRNA fold change following acute aerobic exercise, **(c)** acute resistance exercise and **(d)** inactivity (bed rest or limb immobilization). **(b-d)** Clusters – [a]: gain-of-function increases glucose uptake, [b]: loss-of-function increases glucose uptake, [c]: gain-of-function decreases glucose uptake, [d]: loss-of-function decreases glucose uptake. Red bars indicate increased expression, blue bars is decreased expression (FDR<0.01). Grey bars indicate altered expression with FDR<0.05. **(e)** *Glucose uptake genes* that are regulated by inactivity but reciprocally respond to acute aerobic and/or resistance exercise.

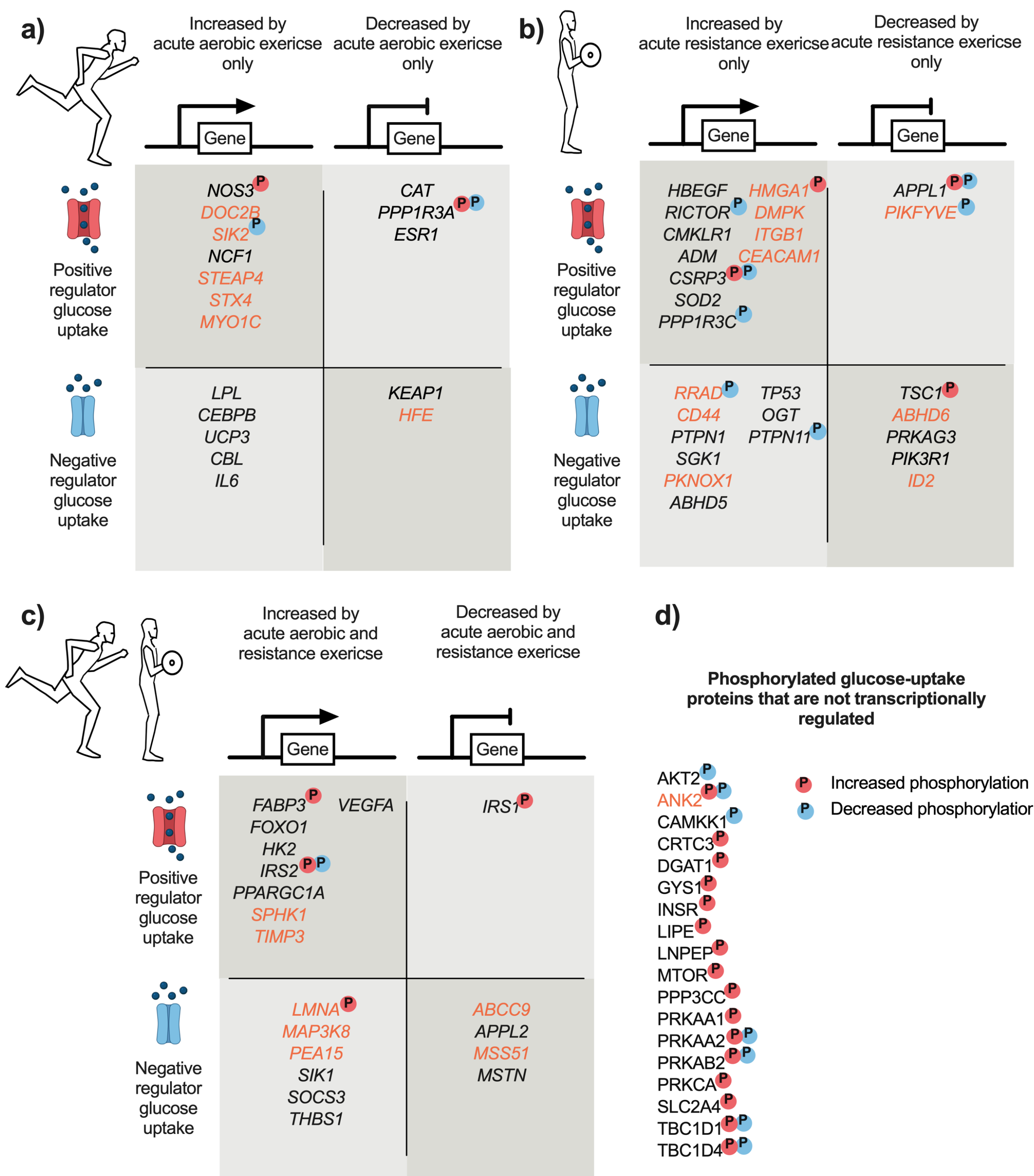


Figure 6. Novel exercise-responsive glucose-uptake genes/proteins. Matrix that categorises *glucose uptake genes* to the variables 'increased vs. decreased expression in response to exercise' and 'positive vs. negative regulator of glucose uptake'. **(a)** *Glucose uptake genes* that are transcriptionally regulated by acute aerobic exercise, **(b)** acute resistance exercise, or **(c)** by both acute aerobic and resistance exercise. Novel targets that are not previously recognised to be regulated by exercise are in orange. Note, positive regulator is when gene gain-of-function increases glucose uptake or when loss-of-function decreases glucose uptake. Negative regulator is when gene gain-of-function decreases glucose uptake (inhibition) or gene loss-of-function increases glucose uptake. **(d)** Overlap of *glucose uptake genes/proteins* with phosphoproteomics datasets to determine targets that

increase (red dot) and decrease (blue dot) phosphorylation in response to human high-intensity bicycling (Nelson et al., 2019), mouse treadmill running (Nelson et al., 2019) and electrically evoked muscle contractions (Potts et al., 2017; Nelson et al., 2019). For targets that are regulated both transcriptionally and by phosphorylation, we indicated increased or decreased phosphorylation in matrices A-C.

4.3 Genes whose gain- or loss-of-function increases endurance capacity

This original systematic review was published in Frontiers in Physiology in May 2019 by Sander Verbrugge, Fakhreddin Yaghoob Nezhad, Martin Schönfelder, Lore Becker, Martin Hrabe de Angelis and Henning Wackerhage. In this systematic review, we identified 31 genes whose gain- or loss-of-function increases endurance capacity in mice.

Yaghoob Nezhad, F., **Verbrugge, S. A.J.**, Schönfelder, M., Becker, L., Hrabě de Angelis, M., & Wackerhage, H. (2019). Genes whose gain or loss-of-function increases endurance performance in Mice: A systematic literature review. Frontiers in Physiology, 10, 262.

Contribution

I am the first author of this manuscript together with Fahkreddin Yaghoob Nezhad and conducted the systematic literature research, collected data from publications and performed bioinformatic analyses. Henning Wackerhage conceptialized the idea behind this manuscript. Together with Henning Wackerhage and Lore Becker, I wrote the first draft. Me together with all other authors contributed to the final submitted manuscript.

Abstract

Endurance is not only a key factor in many sports but endurance-related variables are also associated with good health and low mortality. Twin and family studies suggest that several endurance-associated traits are ≈50% inherited. However, we still poorly understand what DNA sequence variants contribute to endurance heritability. To address this issue, we conducted a systematic review to identify genes whose experimental loss or gain-of-function increases endurance capacity in mice. We found 31 genes including two isoforms of *Ppargc1a* whose manipulation increases running or swimming endurance performance by up to 1800%. Genes whose gain-of-function increases endurance are *Adcy5, Adcy8*, *Hk2, Il15, Mef2c, Nr4a3, Pck1* (Pepck)*, Ppard, Ppargc1a* (both the a and b isoforms of the protein Pgc-1α), *Ppargc1b, Ppp3ca* (calcineurin)*, Scd1, Slc5a7, Tfe3, Tfeb, Trib3* & *Trpv1*. Genes whose loss-of-function increases endurance in mice are *Actn3, Adrb2, Bdkrb2, Cd47, Crym, Hif1a, Myoz1, Pappa, Pknox1, Pten, Sirt4, Thbs1, Thra, and Tnfsf12*. Of these genes, human DNA sequence variants of *ACTN3*, *ADCY5*, *ADRB2*, *BDKRB2*, *HIF1A*, *PPARD*, *PPARGC1A*, *PPARGC1B,* and *PPP3CA* are also associated with endurance capacity and/or VO_2max trainability suggesting evolutionary conservation between mice and humans. Bioinformatical analyses show that there are numerous amino acid or copy number- changing DNA variants of endurance genes in humans, suggesting that genetic variation of endurance genes contributes to the variation of human endurance capacity, too. Moreover, several of these

genes/proteins change their expression or phosphorylation in skeletal muscle or the heart after endurance exercise, suggesting a role in the adaptation to endurance exercise.

Part 2 - First studies on a possible role for the Warburg effect in skeletal muscle hypertrophy

4.4 PKM2 determines myofiber hypertrophy in vitro and increases in response to resistance exercise in human skeletal muscle

This original research article was published in the International Journal of Molecular Sciences by Sander Verbrugge, Sebastian Gehlert, Lian Stadhouders, Daniel Jacko, Thorben Aussieker, Gerard de Wit, Isle Vogel, Carla Offringa, Martin Schönfelder, Richard Jaspers and Henning Wackerhage. In this study we investigated the role of pyruvate kinase (PKM) in myotube hypertrophy and its regulation by resistance exercise. We found that PKM2 determines myotube growth in vitro and increases in response to resistance exercise in human skeletal muscle.

Verbrugge, S.A.J., Gehlert, S., Stadhouders, L.E., Jacko, D., Aussieker, T., MJ de Wit, G., ... & Wackerhage, H. (2020). PKM2 Determines Myofiber Hypertrophy In Vitro and Increases in Response to Resistance Exercise in Human Skeletal Muscle. International journal of molecular sciences, 21(19), 7062.

Contribution

I am the first author of this manuscript. I performed experiments, analysed data from experiments conducted by others and wrote together with Henning Wackerhage and Sebastian Gehlert the draft. All authors contributed to the final submitted manuscript.

Abstract

Nearly 100 years ago, Otto Warburg investigated the metabolism of growing tissues and discovered that tumors reprogram their metabolism. It is poorly understood whether and how hypertrophying muscle, another growing tissue, reprograms its metabolism too. Here, we studied pyruvate kinase muscle (PKM), which can be spliced into two isoforms (PKM1, PKM2). This is of interest, because PKM2 redirects glycolytic flux towards biosynthetic pathways, which might contribute to muscle hypertrophy too. We first investigated whether resistance exercise changes PKM isoform expression in growing human skeletal muscle and found that PKM2 abundance increases after six weeks of resistance training, whereas PKM1 decreases. Second, we determined that *Pkm2* expression is higher in fast compared to slow fiber types in rat skeletal muscle. Third, by inducing hypertrophy in differentiated C2C12 cells and by selectively silencing *Pkm1* and/or *Pkm2* with siRNA, we found that PKM2 limits myotube growth. We conclude that PKM2 contributes to hypertrophy in C2C12 myotubes and indicates a changed metabolic environment within hypertrophying human skeletal muscle fibers. PKM2

is preferentially expressed in fast muscle fibers and may partly contribute to the increased potential for hypertrophy in fast fibers.

5 Discussion

First, I discuss the results from study II and some preliminary findings on PHGDH in primary muscle cells that I have not published yet. Then follows a general discussion with summaries and extension of the discussions I have written for my published manuscripts.

5.1.1 Genes that control glucose uptake in skeletal muscle and their regulation by exercise

We performed a systematic literature review to identify all genes that impact skeletal muscle glucose uptake and cross-referenced these muscle glucose uptake genes to recently generated exercise signaling resources. Notably, over 50% of the muscle glucose uptake genes were regulated by exercise. This concerted exercise signaling comprises several already known regulators of glucose uptake like Calmodulin, CAMKKII, LKB1, AMPK, PKC, RAC1 and GLUT4. Exercise also regulates several classical members of the insulin-signaling cascade for glucose uptake, like TBC1D1, TBC1D4, IRS1, IRS2, FOXO1 and AKT2, highlighting a comprehensive interplay between exercise and insulin signaling that might contribute to how exercise increases insulin sensitivity. The major advancement is that our approach uncovered a host of new links between exercise-responsive and muscle glucose uptake genes. For the discussion below, we refer to these particular glucose uptake genes as *novel candidates*, as these are the genes that are promising candidates to treat insulin resistance of skeletal muscle.

Several novel candidates are linked to the regulation of GLUT4 expression or translocation. GLUT4 facilitates glucose transport into skeletal muscle. The novel candidate DOC2B plays a role in calcium-dependent intracellular vesicle trafficking, including the regulation of GLUT4 fusion into the plasma membrane (Jaldin-Fincati et al., 2017). The latter requires physical interaction with MUNC18 and another novel candidate, STX4, a t-SNARE protein that facilitates docking of GLUT4 to the plasma membrane (Fukuda et al., 2009; Ramalingam et al., 2012). Both DOC2B and STX4 have been studied in the context of insulin signaling and deletion of DOC2B or STX4 resulted in decreased glucose uptake into skeletal muscle (Ramalingam et al., 2012; Yang et al., 2001). Following acute aerobic exercise, these genes are upregulated and their role in exercise-mediated glucose uptake warrants clarification. Another novel candidate is HMGA1. Increasing HMGA1 expression promotes skeletal muscle glucose uptake (Arce-Cerezo et al., 2015). Interestingly, HMGA1 appears to be a positive regulator of insulin receptor expression, while also increasing abundance of GLUT4 in the plasma membrane (Foti et al., 2005). Collectively, this makes it an intriguing finding that acute resistance exercise increases HMGA1 mRNA levels. In contrast, the novel candidate PEA15 seems to be a negative regulator of glucose uptake as global gain-of-function elicits insulin resistance likely due to a decrease in GLUT4 expression and

accompanying decrease in glucose uptake in skeletal muscle (Vigliotta et al., 2004a). Conversely, reducing PEA15 expression stimulates glucose uptake in L6 muscle cells (Ungaro et al., 2012). Surprisingly, skeletal muscle PEA15 is moderately increased in response to both acute aerobic and resistance exercise. Although sustained PEA15 overexpression appears to be detrimental for skeletal muscle glucose uptake, it is remains to be determined how a transient increase in PEA15 affects glucose uptake following exercise.

Other novel candidates relate to angiogenesis. Increased blood flow enhances delivery of glucose to muscle and promotes glucose uptake (Baron et al., 2000). Exercise acutely increase vasodilation but over time also increases capillary density (angiogenesis) in skeletal muscle (Olver and Laughlin, 2016) allowing for greater net blood flow across the muscle bed. CEACAM1, a novel candidate, is a cell adhesion protein that is essential to the formation of new capillaries (Horst et al., 2006). While CEACAM1 loss-of-function decreases muscle glucose uptake in muscle (Park et al., 2006), CEACAM1 expression increases in muscle following exercise which makes it an interesting target to follow-up in the context of exercise training and muscle glucose uptake. Conversely, TIMP3 limits angiogenesis by inhibiting the VEGF receptor (Hanaoka et al., 2014) but its overexpression increases skeletal muscle glucose uptake (Menghini et al., 2012). Interestingly, TIMP3 abundance is elevated in muscle biopsies collected after aerobic and resistance exercise. It is unclear what role TIIMP3 has in exercise-induced glucose uptake. Finally, the novel candidate PKNOX1 is a transcription factor known to promote angiogenesis (Cimmino et al., 2019). Yet, PKNOX1 seems to be a negative regulator of glucose disposal, since loss-of-function increases skeletal muscle glucose uptake (Oriente et al., 2008), while overexpressing PKNOX1 inhibits GLUT4 expression (Ciccarelli et al., 2016; Cimmino et al., 2017). Interestingly, PKNOX1 increases in response to acute resistance exercise and it remains to be clarified how this exercise-induced activation affects angiogenesis, muscle GLUT4 abundance and muscle glucose uptake

Other novel candidates include ID2, a transcription factor that controls glucose and lipid metabolism by interaction with the circadian regulators CLOCK and BMAL1 (Mathew et al., 2013). Disruption of normal diurnal rhythms is linked to an increased risk of developing metabolic disease, including insulin resistance for glucose uptake (Gabriel and Zierath, 2019). Several studies show that exercise adjusts the rhythm of the clock machinery in skeletal muscle, which could be of benefit to glucose metabolism (Gabriel and Zierath, 2019). ID2 is repressed in response to exercise and ID2 loss-of-function increases glucose uptake (Mathew et al., 2013). Thus, ID2 could be an exciting novel link that connect changes in circadian rhythm to metabolic outcome such as improvements in skeletal muscle insulin resistance.

Calcium release from the sarcoplasmic reticulum initiates muscle contractions. Calcium ions are also important secondary messenger that trigger intracellular signaling including cascades that promote skeletal muscle glucose uptake (Endo, 2009; Wright et al.,

2004). The novel candidate RRAD, a regulator of Ca^{2+} channel activity, increases with resistance exercise. RRAD gain-of-function, however, decreases insulin-stimulated glucose uptake (Ilany et al., 2006). Calcium homeostasis is sensitive to flux durations and thus while constitutive overexpression of RRAD is detrimental to glucose uptake, the effects of transient exercise-induced regulation of RRAD requires clarification.

Another novel candidate is ABCC9, which encodes a subunit of potassium channels. Mice that lack ABCC9 (SUR2) show improved insulin action in muscle and glucose uptake (Chutkow et al., 2001b). In agreement, exercise reduces the expression of ABCC9 which could promote glucose uptake. Two other novel candidates are kinases. SPHK1 and DMPK positively regulate glucose uptake (i.e., SPHK1 gain-of-function increases glucose uptake (Bruce et al., 2012), DMPK loss-of-function decreases glucose uptake (Llagostera et al., 2007)). SPHK1 regulates insulin sensitivity by degrading ceramides that could otherwise impair insulin signaling (Bruce et al., 2012). DMPK is involved in Ca^{2+} homeostasis (Botta et al., 2013) and among other functions interacts with HK2 on the mitochondrial membrane participating in neutralizing reactive oxygen species (ROS) (Pantic et al., 2013).

LMNA increases in response to aerobic and resistance exercise and its gain-of-function decreases skeletal muscle glucose uptake (Wojtanik et al., 2009). LMNA is a structural protein of the nuclear membrane, but has also been implicated to regulate gene expression (Hutchison, 2002) including mitochondrial complex I (Boschmann et al., 2010).

Another novel target, MSS51, also controls mitochondrial respiration. MSS51 localizes at the mitochondria where it controls oxidative phosphorylation and fatty acid oxidation (Moyer and Wagner, 2015; Rovira Gonzalez et al., 2019). MSS51 is a key target of MSTN (Moyer and Wagner, 2015), together these proteins are enriched in skeletal muscle (Uhlen et al., 2015) and their loss-of-function increases glucose uptake (Guo et al., 2009; Rovira Gonzalez et al., 2019). MSS51 and MSTN are repressed in response to acute aerobic and resistance exercise. Interestingly, the myostatin inhibitor Bimagrumab, reduces glycated haemoglobin and improves insulin sensitivity in obese individuals (Garito et al., 2018). Like myostatin (MSTN), the other glucose uptake genes we identified serve as potential drug targets that should be explored to treat hyperglycemia and insulin resistance.

Acute endurance-type and resistance-type exercise both improve skeletal muscle insulin resistance (Van Dijk et al., 2012). Yet, interestingly we found in our analysis that 17 glucose-uptake genes are solely responsive to aerobic exercise while 27 specifically responded to resistance exercise, which implies distinct regulation of glucose uptake depending on exercise mode. For example, alterations in iron metabolism genes (STEAP4 and HFE) were specific to acute aerobic exercise. HFE encodes a cell surface protein that binds to the transferrin receptor which regulates the import of iron (Feder et al., 1997). Similarly, STEAP4 colocalizes with transferrin receptor and promotes iron uptake (Ohgami et

al., 2006). Iron is an essential component of mitochondrial complexes and the oxygen-transporting proteins haemoglobin and myoglobin (Davies et al., 1982; Musallam and Taher, 2018) contributing to aerobic exercise performance (Buratti et al., 2015). Interestingly, mice fed a diet enriched with iron had increased skeletal muscle AMPK activity, via a mechanism comprising decreased acetylation of LKB1 which increased LKB1-mediated phosphorylation of $AMPK^{Thr172}$ and accompanying glucose uptake in skeletal muscle (Huang et al., 2013). HFE is repressed by acute aerobic exercise and loss-of-function of HFE increases both glucose uptake and AMPK phosphorylation (Huang et al., 2007). In contrast, STEAP4 is induced by exercise and loss-of-function decreases glucose uptake (Wellen et al., 2007). This indicates that both HFE and STEAP4 are positive regulators of exercise-induced glucose uptake and iron metabolism might herein play an unrecognized role.

Certain genes involved in producing and combatting oxidative stress including NCF1, KEAP1 and CAT are only altered in response to acute aerobic exercise. As part of the NADPH oxidase complex (NOX2), NCF1 contributes to cytosolic superoxide production in skeletal muscle during contractions (Henríquez-Olguin et al., 2019; Sakellariou et al., 2013). Also in the cytosol, KEAP1 acts as a sensor for oxidative stress promoting expression of antioxidants including catalase (CAT) that neutralizes hydrogen peroxide by converting it to water and oxygen (Seidel et al., 2019). Acute resistance exercise, on the other hand, induces different genes that combat oxidative stress which are localized on the mitochondria. These include SOD2 which binds superoxide to produce hydrogen peroxide (Klug et al., 1972; Miller, 2012) and DMPK that regulates mitochondrial membrane permeability (Pantic et al., 2013). Still, DMPK is also present in the cytosol where it has been suggested to participate in oxidative stress regulation (Pantic et al., 2013). For example, DMPK interacts with the small GTPase RAC1 (Shimizu et al., 2000) that is necessary for oxidant production in skeletal muscle and subsequent exercise-stimulated-glucose uptake (Henríquez-Olguin et al., 2019; Sylow et al., 2013a). RAC1 is mechanosensitive and mediates mechanical stress-induced glucose uptake (Sylow et al., 2015). Resistance exercise causes mechanical stress (Wackerhage et al., 2019), which promotes glucose uptake (Jensen et al., 2014). We found that resistance exercise increased the expression of genes that are associated with mechanotransduction or sarcomere structure, including the novel candidates ITGB1 and DMPK, but also the little known target CSRP3 (Kostek et al., 2007; Ravel-Chapuis et al., 2017; Vafiadaki et al., 2015; Zhang et al., 2007). Finally, we identified resistance-exercise specific regulation of PIKFYVE and ABHD6 which play a role in the synthesis of GLUT4 storage vesicles. PIKFYVE, downstream of PI3K, controls the formation of GLUT4 storage vesicles (i.e. endosomes) (Shisheva, 2008)and accordingly its loss-of-function decreases glucose uptake (Ikonomov et al., 2013). Before, Sylow et al. (2017) suggested that PIKFYVE could be involved in exercise-stimulated glucose uptake and this still remains to be determined (Sylow et al., 2017).

Formation of GLUT4 storage vesicles could be supported by another novel target, ABHD6, a lipase that co-localizes with endosomes in liver to provide lipids for vesicle membranes (Pribasnig et al., 2015). However, whether ABHD6 plays a similar role in skeletal muscle remains obscure. Its expression in skeletal muscle is decreased following exercise, but loss-of-function promotes glucose uptake (Zhao et al., 2016a). This contradiction might be explained by global loss-of-function that also targets pancreas where ABHD6 modulates insulin secretion (Zhao et al., 2016a).

Although our focus was on skeletal muscle glucose uptake in response to exercise as environmental stimulus, diabetes is known to be partially inherited which makes some individuals more prone to diabetes than others (Hemminki et al., 2010). Genome-wide association studies (GWAS) and sequencing studies have identified common and rare DNA variants in genes that associate with T2D (Fuchsberger et al., 2016; Morris et al., 2012). Whether these DNA variations in certain genes play a role in skeletal muscle glucose uptake is often unknown. Here, complementary research in transgenic mice has identified genes that regulate glucose uptake in muscle (Deems et al., 1994; Zisman et al., 2000). Previously identified DNA variants that associate with diabetes in humans overlap with our gene list, including BCL2, DGKB, IRS1, KCNJ11, PPARG, TBC1D4 (Flannick and Florez, 2016). Our compiled list of 176 genes can serve as candidates for targeted genetic analyses to discover additional DNA variants that associate with hyperglycemia, insulin resistance and diabetes.
Acute resistance exercise is beneficial for glucose homeostasis by reducing hyperglycemic episodes (Van Dijk et al., 2012; Marcus et al., 2013b) whereas long term resistance training is believed to increase the capacity for glucose disposal by gaining muscle mass (Wolfe, 2006).

We have previously proposed that increased glucose uptake in response to acute resistance exercise provides carbon for synthesizing amino acids, nucleotides and lipids to support anabolism in hypertrophying muscles (Verbrugge et al., 2020). Recently, glycolysis has been shown to limit basal protein synthesis (Suginohara et al., 2021) suggesting that glucose metabolism plays a role in the maintenance of muscle mass. The manipulation of several glucose uptake genes are associated with regulating muscle hypertrophy and glucose uptake in concert. For example, MSTN loss-of-function is known to increase muscle mass (McPherron et al., 1997) while simultaneously increasing glucose uptake (Guo et al., 2009). Other examples are *Foxc2, Fst, Ccn5, Cpt1b, Hdac3, Insr, Steap4, Igfbp3, Tnfsf11, Inpp5k* and *Nos2*, whose gene manipulation affect both muscle weight and glucose uptake. Many of the studies that looked at glucose uptake after gene manipulation did not assess muscle weight or cross-sectional area. It would be interesting to find out which of the 176 glucose uptake genes also control muscle mass. The genes we identified serve as candidates to study

the interplay between glucose metabolism and skeletal muscle hypertrophy to discover targets for treatment of sarcopenia and insulin resistance.

5.1.2 PHGDH function in primary skeletal muscle cells

The work for my dissertation consists of systematic reviews and experimental data on Warburg effect-associated enzymes. Since I have not published my data on PHGDH in primary skeletal muscle stem cells previously, here follows a discussion of my experimental findings. In preliminary experiments, I determined that during embryogenesis PHGDH is widely expressed in different tissues (not shown). Homozygous mutation of *PHGDH* in humans results in Neu-Laxova syndrome manifesting in prenatal death, structural deformities of the central nervous system and brain, skin abnormalities, flexed limbs, and atrophic and underdeveloped muscles (El-Hattab et al., 2016; Shved et al., 1985). A complete loss-of-function of *Phgdh* in mice shows the same features including prenatal death, reduced brain and body size(Yoshida et al., 2004). This illustrates the indispensable role of Phgdh during early development.

Although PHGDH abundance is low in adult skeletal muscle, it is indispensable for myotube growth (Stadhouders et al., 2020). This suggests that PHGDH is important during formation and maturation of myotubes. In preliminary experiments, I found that PHGDH is highest expressed during proliferation but not abundant in quiescence (not shown). This is in agreement with previous RNA sequencing data where cultured muscle stem cells show increased Phgdh expression (7.7-fold) compared to freshly isolated muscle stem cells (e.g quiescence) (Ryall et al., 2015). Increased *Phgdh* expression was also evident in cardiotoxin injured mouse skeletal muscle, where proliferation of muscle stem cells is high (Lukjanenko et al., 2013). This suggests that PHGDH plays a role in myoblast proliferation and possibly limits muscle regeneration. Future studies should apply the cardiotoxin injury model in muscle stem cell specific PHGDH knock-out mice.

Finally, in our lab we observed that PHGDH goes to the nucleus during differentiation where it has an unknown function. Glycolytic enzymes also localize in the nucleus, where they regulate cell proliferation, or contribute to autophagy and DNA repair. For example, HK2 participates in gene transcription (Sheikh et al., 2018) and GAPDH can translocate from cytosol to the nucleus to initiate apoptosis (Hara et al., 2005) or form a binding complex with LDH that regulates cell cycle progression (Zheng et al., 2003). Also, pyruvate kinase is well established to have functions in the nucleus (Zhang et al., 2019). Future studies should focus on co-immunoprecipitation of PHGDH to identify binding partners. This will give us insight in possible mechanism for translocation of PHGDH from cytosol to nucleus during differentiation and thereby delineating PHGDH’s nuclear function.

5.2 General discussion

Having sufficient skeletal muscle mass is important for health (Wolfe, 2006). How much muscle mass we have depends the external stimuli our muscles are exposed to (nurture) and the DNA sequence of our genome (nature). The aim of this dissertation was to investigate whether glucose metabolism contributes to skeletal muscle hypertrophy. I conducted a systematic review and identified 47 *hypertrophy genes* whose gain- or loss-of-function increases muscle mass in mice (Verbrugge et al., 2018). Many of these genes are involved in common signalling including IGF1-AKT-MTOR or myostatin-Smad. Both these pathways are known to regulate muscle mass, but also glucose metabolism (Akasaki et al., 2014; Guo et al., 2009; McPherron et al., 1997; Rommel et al., 2001).

Glucose uptake is another trait that depends on nature and nurture (Van Dijk et al., 2012; Zisman et al., 2000) and is affected by skeletal muscle mass (Wolfe, 2006). Unfortunately, most studies that investigate the role of gene knockout or overexpression do not routinely assess skeletal muscle glucose uptake. To find out what genes regulate skeletal muscle glucose uptake I conducted a second systematic review and identified 176 genes whose manipulation alters glucose uptake in mice. Twelve of those genes control both glucose uptake and muscle mass, indicating that muscle hypertrophy could depend on glucose metabolism. This is likely an underestimation since most studies did not investigate muscle size, or did not mention it.

Glucose is taken up in skeletal muscle via GLUT4 (Zisman et al., 2000) where it is phosphorylated by hexokinase and enters glycolysis (Chang et al., 1996). It has been proposed that glucose contributes to anabolism (Lunt and Vander Heiden, 2011) possibly through pyruvate kinase muscle isoform 2 (PKM2) (Christofk et al., 2008). I found that PKM2 is more abundant after 6 weeks of resistance exercise together with muscle mass regulators AKT and P70S6K (Verbrugge et al., 2020). On the other hand, PKM1 was less abundant suggesting a possible isoform shift from PKM1 to PKM2 in growing skeletal muscle which has before been associated with increased anabolism in cancer cells (Christofk et al., 2008).

Phosphoglycerate dehydrogenase (PHGDH) branches from glycolysis and directs glucose into the *de novo* serine synthesis pathway (Greenberg and Ichihara, 1957) I found that PHGDH is present in most embryonic mouse tissues at 12.5 days post coitus, but that expression in adult skeletal muscle is negligible. On the other hand, PHGDH is expressed in activated muscle stem cells. Muscle stem cells express PHGDH during proliferation *in vitro*, after which levels decreases upon differentiation. This suggests that PHGDH is more involved during early myogenesis (e.g embryonic development) where myoblasts actively proliferate, then in differentiated skeletal muscle.

5.2.1 Genes that regulate glucose uptake and skeletal muscle hypertrophy

Acute resistance exercise regulates the transcription of > 2400 genes (Pillon et al., 2020). The pulsatile gene expression in response to exercise leads over time to muscle adaptations (Egan and Zierath, 2013). Although we know that inhibition of MTOR prevents resistance exercise-induced protein synthesis necessary for gains in muscle mass (Drummond et al., 2009) little is known about other candidates that control resistance exercise induced muscle growth.

Many of the *glucose uptake genes* we identified respond to acute resistance exercise. We have previously proposed that increased glucose uptake in response to acute resistance exercise provides carbon for synthesizing amino acids, nucleotides and lipids to support anabolism in hypertrophying muscles (Verbrugge et al., 2020). The manipulation of genes associated with regulating muscle hypertrophy do also control glucose uptake in concert including *Mstn, Foxc2, Fst, Ccn5, Cpt1b, Hdac3, Insr, Steap4, Igfbp3, Tnfsf11, Inpp5k* and *Nos2.* These targets were before not recognised to regulate both muscle mass and glucose uptake.

Resistance exercise increases protein synthesis and glycolytic enzyme expression (Atherton and Smith, 2012; Pillon et al., 2020). Glycolysis limits basal protein synthesis (Suginohara et al., 2021) showing that glucose metabolism contributes to maintaining muscle mass. Disrupted glycolysis leads to exercise intolerance and fatigue, indicating its critical role in energy metabolism (DiMauro et al., 1982; García et al., 2009; Servidei et al., 1986; Tarui et al., 1965). But genetic studies have also revealed that glycolysis contributes to tissue growth and development.

Mice with liver-specific disruption of the *Gck* gene (also known as *Hk4*) show decreased body weight 23%, but ~30% increased ventricular wall thickness (Tsukita et al., 2012). A loss-of-function of *Gck* in Sf1 neurons decreases white adipose tissue (Steinbusch et al., 2016), whereas knock-out of *Aldob* increases lipid deposits (Oppelt et al., 2015). Knock-out of *Pfkm* decreases fat mass on average by ~70% (Getty-Kaushik et al., 2010), and *Pfkl* overexpression increases body weight (Knobler et al., 1997). *Pkm2* loss-of-function increases liver size but muscle mass remains unaffected (Dayton et al., 2016). Little is known about the role of glycolysis in muscle mass regulation. In the fruit fly (*Drosophila melanogaster*), loss-of-function of the lactate producing enzyme *Imp3* (*Ldha* orthologue) results in muscles that have more, but thinner fibers (Tixier et al., 2013). Muscle specific knock-out of other glycolytic enzymes (*Pgam2* orthologue, *Pfk, Tpi, Gapdh, Pgk* and *Pk* orthologue) result in a similar phenotype with a reduced capacity to form actin (Tixier et al., 2013).

The fact that glycolysis is indispensable for life makes uncovering their tissue-specific roles challenging. Indeed, Glycolytic is essential for life and a loss-of-function of hexokinase (*Gck* or *Hk2*), for example, is embryonically lethal (Grupe et al., 1995; Wu et al., 2012).

Homozygous loss-of-function of aldolase (*Aldob) or phosphofructokinase* (*Pfkm)* results in high mortality rates (García et al., 2009; Oppelt et al., 2015).

Similarly, high mortality rates complicates the study of PHGDH, where crossing heterozygote Phgdh null mice creates retarded fetuses and does not give viable homozygote Phgdh null offspring (Yoshida et al., 2004). A complete loss-of-function of Phgdh results in prenatal death, reduced brain and body size, and lower circulating serine concentrations. Yoshida et al. (2004) did not analyse muscle development in detail in the Phgdh deficient embryos. They do however report that somites seem to develop in the Phgdh null embryos, whereas the limb bud do show 'abnormalities' (Yoshida et al., 2004).Embryo heads devote of Phgdh show reduced expression of genes important for muscle development as well as protein translation (Furuya et al., 2008; Sayano et al., 2013). Upon IGF1 exposure, Phgdh mRNA increases in C2C12 myotubes (Adams, 2007) suggesting that Phgdh is important for the growth response. Likewise, Brown et al. (2016) observed increased expression of Phgdh after inducing hypertrophy in fully differentiated pig muscle (Brown et al., 2016). Finally, knockdown of PHGDH with siRNA reduces myotube size in hypertrophying myotubes (Stadhouders et al., 2020). Together, this indicates that PHGDH contributes to muscle growth, but has to be validated in vivo. Inducible muscle-specific gene manipulation studies are necessary to circumvent the lethality problem and investigate the contribution of glycolytic enzymes and PHGDH to skeletal muscle hypertrophy.

This applies also to the other glycolytic enzyme I studied, PKM2. I showed that PKM2 abundance is increased in growing skeletal muscle after 6 weeks of resistance exercise. Disrupting Pkm2 mRNA in C2C12 myotubes abolishes the hypertrophic response which indicates its importance for muscle growth. Interestingly, the loss-of-function of Pkm2 increases liver size but muscle mass remains unaffected (Dayton et al., 2016). These mice, however, compensate for a loss of Pkm2 by upregulating Pkm1 expression during embryogenesis. It is also not clear how these mice would respond to a muscle hypertrophy stimulus, since muscle mass was measured in sedentary mice. PKM2 is regulated by AKT (Salani et al., 2015) which controls both muscle mass and glucose metabolism (Akasaki et al., 2014). Inducing Akt activation in Pkm2-defient mice would allow us to asses if these mice develop muscle hypertrophy despite a lack of PKM2.

5.2.3 Limitations

Systematic reviews have clear strengths such as providing an overview of the current state of scientific literature, and the opportunity to discover relevant targets that can be follow-up with experiments. But also some limitations apply. First, the list of genes we identified that control glucose uptake, skeletal muscle mass and endurance capacity is not exhaustive. Animal

models allows us to study the effect of every single gene in the genome, but until they are available for all genes, we can't compile a complete list of all candidates that regulate glucose uptake, skeletal muscle mass and endurance capacity.

Second, it is possible that several of the *hypertrophy genes* and *glucose uptake genes* control both muscle mass and glucose metabolism, but we have overlooked. We only assessed the original papers that report an effect on muscle mass or glucose uptake after gene gain or loss- of-function. A follow-up literature evaluation for the identified candidate genes was not performed. Skeletal muscle mass and glucose uptake are often not assessed in the same study. Extending literature research beyond the first-mention studies could extend the number of genes that regulate both skeletal muscle mass or glucose uptake.

Third, the inclusion and exclusion criteria in a systematic review determine which studies are assessed and included in the analysis. These strict criteria might have led us to miss relevant studies. For example, we did not include studies where muscle hypertrophy or glucose uptake was accompanied by a pathological phenotype. In this case the manipulated gene can still play a role in muscle hypertrophy or glucose metabolism, but did not meet our criteria for inclusion and therefore not assessed for the systematic reviews.

We have conducted observational and in vitro studies to investigate the role of PKM2 and PHGDH in muscle hypertrophy. The strength of this approach is that we determined altered PKM2 protein abundance from muscle biopsies following resistance training, indicating a physiological role in muscle adaptation. We followed-up with in vitro experiments in isolated muscle cells, which is not confounded by other cells types such as fibroblasts that are present in muscle biopsies. In isolated muscle cells PKM2 and PHGDH are essential for myotube growth. But the main limitation is that we did not have an animal model available to validate the role of PKM2 and PHGDH in skeletal muscle hypertrophy. We are currently working on muscle-specific PHGDH knock-out mice, that gives us the opportunity to establish the importance of PHGDH under growth stimulated conditions in vivo. For PKM2, future studies need to verify its role in muscle mass regulation in inducible PKM2-specific knockout mice.

5.2.4 Future directions

The findings from the work in my dissertation lead to several new research questions that remain to be answered:

- Can genes from systematic review be targeted to modulate glucose uptake and skeletal muscle hypertrophy?
- How does PKM2 and PHGDH limit myotube growth?
- Is glucose flux altered by PKM2 or PHGDH disruption in vitro?

- Is PHGDH essential for muscle regeneration?

To answer these questions, candidates from our systematic reviews can be followed-up to study the interplay between skeletal muscle growth and glucose metabolism. To elucidate the mechanism by which PKM2 and PHGDH limit myotube growth, carbon isotope tracing in glucose molecules offers the opportunity to determine if PHGDH and PKM2 direct glucose flux in anabolic pathways. This can be done in vitro and in vivo. Furthermore, in vivo experiments in skeletal muscle specific PKM2 and PHGDH mouse models lets us validate their role in skeletal muscle hypertrophy. Finally, PHGDH is highest expressed in proliferating muscle stem cells. This suggests that PHGDH is important for muscle regeneration when proliferation is high. A mouse line where PHGDH is specifically knocked-out in muscle stem cells is currently being developed and allows us to study whether PHGDH limits muscle regeneration.

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Appendix

Supplemental data

Table S1. Difference basal glucose uptake after gene manipulation. Overview of genes whose gain or loss-of-function alters basal glucose uptake. Indicated in table is the study (author), manipulated gene, whether manipulation was gain or loss-of-function, average glucose uptake (of all measured muscles) and effect on muscle mass.

Author	***Gene***	**GAIN or LOSS of function**	**Average difference glucose uptake**	**Effect on muscle mass**
(Marshall et al., 1993)	*Slc2a1*	GAIN	330%	Not specified
(Pospisilik et al., 2007)	*Aifm1*	LOSS	290%	Not specified
(Witczak et al., 2007)	*Camkk1*	GAIN	150%	Not specified
(Hajri et al., 2002)	*Cd36*	LOSS	130%	Not specified
(Wende et al., 2007)	*Ppargc1a*	GAIN	130%	Not specified
(Molero et al., 2004)	*Cbl*	LOSS	120%	Not specified
(Kang et al., 2012)	*Mcat*	GAIN	115%	Not specified
(Zong et al., 2011)	*Vamp8*	LOSS	110%	No effect
(Ahn et al., 2019)	*Mlxip*	LOSS	105%	Not specified
(Song et al., 2010)	*Crtc3*	LOSS	105%	Not specified
(Hofmann et al., 2007)	*Lrp1*	LOSS	100%	Not specified
(Yokoyama et al., 2014)	*Tp53*	LOSS	95%	No effect
(Hoffmann et al., 2020)	*Bmp4*	GAIN	90%	Not specified
(Rajkumar et al., 1996)	*Igfbp1*	GAIN	83%	Not specified
(Risson et al., 2009)	*Mtor*	LOSS	80%	Decrease
(Klaman et al., 2000)	*Ptpn1*	LOSS	75%	No effect
(Taddeo et al., 2014)	*Ppif*	LOSS	75%	Not specified
(Ryder et al., 2003)	*Ppp3cc*	LOSS	70%	Not specified
(Xie et al., 2015)	*Abhd5*	LOSS	70%	No effect
(Radović et al., 2016)	*Lipa*	LOSS	70%	Not specified
(Shi et al., 2018)	*Ogt*	LOSS	67%	No effect

(Levin et al., 2007)	*Dgat2*	GAIN	67%	Not specified
(Inoue et al., 2013)	*Thbs1*	LOSS	52%	Not specified
(Miki et al., 2002)	*Kcnj11*	LOSS	52%	Not specified
(Boini et al., 2006)	*Sgk1*	LOSS	50%	Not specified
(Zhao et al., 2016)	*Abhd6*	LOSS	50%	Not specified
(Fischer et al., 2019)	*Pid1*	LOSS	50%	Not specified
(Mathew et al., 2013)	*Id2*	LOSS	45%	Not specified
(Chang et al., 1996)	*Hk2*	GAIN	45%	Not specified
(Sydow et al., 2008)	*Ddah1*	GAIN	45%	Not specified
(Guo et al., 2009)	*Mstn*	LOSS	40%	Increase
(Uruno et al., 2016)	*Keap1*	LOSS	40%	Not specified
(Kee et al., 2015)	*Tpm3*	GAIN	40%	Not specified
(Lee et al., 2007)	*Ptprv*	LOSS	35%	Not specified
(Sugita et al., 2011)	*Rxrg*	GAIN	35%	Decrease
(Terauchi et al., 1999)	*Pik3r1*	LOSS	33%	Not specified
(Fritah et al., 2012)	*Nrip1*	LOSS	30%	Not specified
(Xu et al., 2004)	*Kcna3*	LOSS	25%	Not specified
(Tao et al., 2015)	*Ggps1*	LOSS	20%	No effect
(Huang et al., 2007)	*Hfe*	LOSS	18%	Not specified
(Agudo et al., 2010)	*Cnr2*	LOSS	-20%	Not specified
(Zhou et al., 2017)	*Rab28*	LOSS	-20%	Not specified
(Lundell et al., 2019)	*Foxo3*	LOSS	-20%	Not specified
(Erol et al., 2004)	*Fabp3*	LOSS	-27%	Not specified
(Silha et al., 2002)	*Igfbp3*	GAIN	-28%	Decrease
(Keller et al., 2002)	*Lnpep*	LOSS	-30%	No effect
(Crosson et al., 2003)	*Ppp1r3c*	LOSS	-30%	Not specified
(Pederson et al., 2005)	*Gys1*	LOSS	-33%	No effect
(Hennige et al., 2010)	*Prkcb*	GAIN	-34%	Not specified
(Aoi et al., 2019)	*Sparc*	LOSS	-35%	Not specified
(Lundell et al., 2019)	*Foxo1*	LOSS	-35%	Not specified

(Duplain et al., 2001)	*Nos3*	LOSS	-40%	Not specified
(Pedersen et al., 2007)	*Cebpa*	LOSS	-40%	Not specified
(Ernst et al., 2012)	*Cmklr1*	LOSS	-40%	No effect
(Kee et al., 2015)	*Tpm3*	LOSS	-40%	Not specified
(Fritah et al., 2012)	*Nrip1*	GAIN	-45%	Not specified
(Fisette et al., 2013)	*C5ar2*	LOSS	-49%	Not specified
(Tang et al., 2017)	*Chga*	LOSS	-50%	Decrease
(Delibegovic et al., 2003)	*Ppp1r3a*	LOSS	-69%	Not specified
(Park et al., 2014)	*Sik2*	LOSS	-70%	Not specified

Table S2. Difference insulin-stimulated glucose uptake after gene manipulation. Overview of genes whose gain or loss-of-function alters insulin-stimulated glucose uptake. Indicated in table is the study (author), manipulated gene, whether manipulation was gain or loss-of-function, average glucose uptake (of all measured muscles) and effect on muscle mass.

Author	Gene	GAIN or LOSS	Average difference glucose uptake	Effect on muscle mass
(Kang et al., 2011)	*Itga2*	LOSS	313%	Not specified
(Oriente et al., 2008)	*Pknox1*	LOSS	245%	Not specified
(Hasib et al., 2019)	*Cd44*	LOSS	185%	Not specified
(Koh et al., 2013)	*Trib3*	LOSS	180%	Not specified
(Bilanges et al., 2017)	*Pik3c3*	LOSS	145%	No effect
(Marshall et al., 1993)	*Slc2a1*	GAIN	135%	Not specified
(Ijuin et al., 2008)	*Inpp5k*	LOSS	110%	Increase
(Kang et al., 2016)	*Ilk*	LOSS	108%	No effect
(Senagolage et al., 2018)	*Bcl6*	LOSS	98%	No effect
(Anderson et al., 2009)	*Cat*	GAIN	95%	Not specified
(Kim et al., 2005)	*Foxc2*	GAIN	90%	Increase
(Neschen et al., 2008)	*Ucp1*	GAIN	90%	Decrease
(Nixon et al., 2016)	*Sik1*	LOSS	85%	Not specified
(Qi et al., 2011)	*Ghsr*	LOSS	83%	Not specified
(Ayala et al., 2009)	*Glp1r*	LOSS	83%	Not specified
(Zong et al., 2011)	*Vamp8*	LOSS	83%	No effect
(Agudo et al., 2010)	*Cnr2*	LOSS	80%	Not specified
(Perfield et al., 2011)	*Map3k8*	LOSS	80%	Not specified
(Takahashi et al., 2011)	*Rarres2*	LOSS	80%	Not specified
(Fukatsu et al., 2009)	*Hbegf*	GAIN	79%	No effect
(Xiao et al., 2010)	*Sirt6*	LOSS	75%	Not specified
(Ryder et al., 2003)	*Ppp3cc*	LOSS	71%	Not specified
(Han et al., 2019)	*Fst*	GAIN	71%	Increase
(Zhang et al., 2011)	*Mapk8*	LOSS	70%	Not specified

(Deems et al., 1994)	*Slc2a4*	GAIN	70%	Not specified
(Menghini et al., 2012)	*Timp3*	GAIN	70%	Not specified
(Hui et al., 2008)	*Txnip*	LOSS	68%	Not specified
(Purcell et al., 2011)	*Slc2a12*	GAIN	68%	Not specified
(Seyer et al., 2013)	*Slc2a2*	LOSS	65%	No effect
(Kee et al., 2015)	*Tpm3*	GAIN	60%	Not specified
(Lee et al., 2014)	*Camk4*	GAIN	60%	No effect
(Chutkow et al., 2001)	*Abcc9*	LOSS	59%	Not specified
(Xu et al., 2004)	*Kcna3*	LOSS	55%	Not specified
(Cheng et al., 2014)	*Appl2*	LOSS	55%	Not specified
(Schulz et al., 2016)	*Bmpr1a*	LOSS	55%	Not specified
(Wang et al., 2000)	*Cebpb*	LOSS	54%	Not specified
(Grünberg et al., 2017)	*Ccn5*	GAIN	54%	Increase
(Ahn et al., 2019)	*Mlxip*	LOSS	50%	Not specified
(Rovira Gonzalez et al., 2019)	*Mss51*	LOSS	50%	No effect
(Chang et al., 1996)	*Hk2*	GAIN	49%	Not specified
(Bruce et al., 2012)	*Sphk1*	GAIN	47%	No effect
(Sylow et al., 2013b)	*Rac1*	LOSS	45%	Not specified
(Terauchi et al., 1999)	*Pik3r1*	LOSS	40%	Not specified
(Jorgensen et al., 2013)	*Socs3*	LOSS	40%	Not specified
(Wijesekara et al., 2005)	*Pten*	LOSS	40%	No effect
(Arce-Cerezo et al., 2015)	*Hmga1*	GAIN	40%	No effect
(Birkenfeld et al., 2011)	*Slc13a5*	LOSS	38%	Not specified
(Boini et al., 2006)	*Sgk1*	LOSS	38%	Not specified
(Chen et al., 2006)	*Ucn2*	LOSS	38%	Not specified
(Ropelle et al., 2013)	*Nos2*	LOSS	38%	Increase
(Leitges et al., 2002)	*Prkca*	LOSS	35%	Not specified
(Yu et al., 2001)	*Gnas*	LOSS	33%	Not specified
(Hong et al., 2009)	*Il10*	GAIN	30%	No effect

(Zhao et al., 2016)	*Abhd6*	LOSS	30%	Not specified
(Funai et al., 2016)	*Cept1*	LOSS	30%	No effect
(Guridi et al., 2015)	*Tsc1*	LOSS	30%	Not specified
(Bartels et al., 2014)	*Apob*	GAIN	30%	Not specified
(Liu et al., 2007)	*Dgat1*	GAIN	30%	Not specified
(Toyoda et al., 2011)	*Myo1c*	GAIN	20%	Not specified
(Szekeres et al., 2012)	*Tbc1d1*	LOSS	19%	No effect
(Barnes et al., 2005)	*Prkag3*	LOSS	15%	Not specified
(Cheng et al., 2014)	*Appl1*	GAIN	15%	Not specified
(Maddux et al., 2006)	*Enpp1*	GAIN	13%	Not specified
(Lorenzo et al., 2015)	*Ank2*	LOSS	1%	Not specified
(Barnes et al., 2005)	*Prkag3*	GAIN	0%	Not specified
(Fernández et al., 2001)	*Igf1r*	LOSS	-3%	Not specified
(Rodriguez-Araujo et al., 2015)	*Snca*	LOSS	-10%	Not specified
(Mulder et al., 2003)	*Lipe*	LOSS	-17%	Not specified
(Kumar et al., 2008)	*Rictor*	LOSS	-20%	Not specified
(Steinberg et al., 2010)	*Prkab2*	LOSS	-20%	Not specified
(Liu et al., 2007)	*Dgat1*	LOSS	-20%	Not specified
(Rajkumar et al., 1996)	*Igfbp1*	GAIN	-20%	Not specified
(Maegawa et al., 1999)	*Ptpn11*	GAIN	-20%	Not specified
(Bonner et al., 2013)	*Vegfa*	LOSS	-23%	No effect
(Ilany et al., 2006)	*Rrad*	GAIN	-24%	Not specified
(Dyar et al., 2014)	*Arntl*	LOSS	-25%	Not specified
(Watt et al., 2017)	*Inppl1*	LOSS	-25%	No effect
(Hernandez-Carretero et al., 2018)	*Csrp3*	LOSS	-25%	Not specified
(Wojtanik et al., 2009)	*Lmna*	GAIN	-25%	Increase
(Keller et al., 2002)	*Lnpep*	LOSS	-29%	No effect
(Hevener et al., 2003)	*Pparg*	LOSS	-30%	Not specified

(Llagostera et al., 2007)	*Dmpk*	LOSS	-30%	Not specified
(Hong et al., 2007)	*Ins2*	LOSS	-30%	No effect
(Levin et al., 2007)	*Dgat2*	GAIN	-30%	Not specified
(Wang et al., 2003)	*Ucp3*	GAIN	-30%	Not specified
(Cheng et al., 2014)	*Appl2*	GAIN	-30%	Not specified
(Bryzgalova et al., 2006)	*Esr1*	LOSS	-33%	Not specified
(Shimosawa et al., 2003)	*Adm*	LOSS	-33%	Not specified
(Lantier et al., 2018)	*Sirt2*	LOSS	-33%	Not specified
(Kramer et al., 2006)	*Tbc1d4*	GAIN	-33%	Not specified
(Moller et al., 1996)	*Insr*	LOSS	-35%	Decrease
(Dhar et al., 2006)	*Atp10a*	LOSS	-35%	Not specified
(Groh et al., 2009)	*Sgcb*	LOSS	-35%	Increase
(Kubota et al., 2011)	*Irs2*	LOSS	-35%	Not specified
(Ganesh Kumar et al., 2012)	*Enho*	LOSS	-35%	Not specified
(Sugita et al., 2011)	*Rxrg*	GAIN	-35%	Decrease
(Park et al., 2006)	*Ceacam1*	GAIN	-37%	No effect
(Kang et al., 2014)	*Mmp9*	LOSS	-38%	Increase
(Franckhauser et al., 2008)	*Il6*	GAIN	-38%	Not specified
(Yakar et al., 2004)	*Igf1*	LOSS	-39%	Not specified
(Ikonomov et al., 2013)	*Pikfyve*	LOSS	-39%	No effect
(Duplain et al., 2001)	*Nos3*	LOSS	-40%	Not specified
(Raciti et al., 2011)	*Ankrd26*	LOSS	-40%	Not specified
(Vigliotta et al., 2004)	*Pea15*	GAIN	-40%	Not specified
(Villena et al., 2008)	*Dlk1*	GAIN	-40%	No effect
(Spurlin et al., 2003)	*Stxbp3*	GAIN	-42%	No effect
(Zong et al., 2009)	*Itgb1*	LOSS	-43%	Not specified
(Aoi et al., 2019)	*Sparc*	LOSS	-45%	Not specified
(Wellen et al., 2007)	*Steap4*	LOSS	-45%	Decrease
(Gao et al., 2009)	*Cpt1c*	LOSS	-45%	Not specified
(Huang et al., 2011)	*Col5a3*	LOSS	-45%	Not specified

(Manrique et al., 2012)	*Esr1*	LOSS	-45%	Not specified
(Ramalingam et al., 2012)	*Doc2b*	LOSS	-45%	Not specified
(Lantier et al., 2015)	*Sirt3*	LOSS	-45%	Not specified
(Bonnet et al., 2019)	*Tnfsf11*	GAIN	-45%	Decrease
(Kim et al., 2001)	*Lpl*	GAIN	-48%	Not specified
(Park et al., 2014)	*Sik2*	LOSS	-50%	Not specified
(Yang et al., 2001)	*Stx4*	LOSS	-50%	Not specified
(Zabolotny et al., 2001)	*Ptprf*	GAIN	-50%	Not specified
(Cooksey et al., 1999)	*Gfpt1*	GAIN	-50%	Not specified
(Farese et al., 2007)	*Prkci*	LOSS	-53%	Not specified
(Cho et al., 2001)	*Akt2*	LOSS	-55%	Not specified
(Kalupahana et al., 2012)	*Agt*	GAIN	-55%	Not specified
(Yamauchi et al., 1996)	*Irs1*	LOSS	-60%	Not specified
(Oshikawa et al., 2004)	*Cav3*	LOSS	-60%	Not specified
(Kim, 2014)	*Cpt1b*	LOSS	-60%	Decrease
(Gangadhariah et al., 2017)	*Cyp2c23*	LOSS	-60%	Increase
(Chadt et al., 2008)	*Nob1*	GAIN	-60%	Not specified
(Li et al., 2014)	*Arfgef3*	LOSS	-65%	Not specified
(Hong et al., 2017)	*Hdac3*	LOSS	-70%	Decrease

Table S3. Difference contraction/exercise-stimulated glucose uptake after gene manipulation. Overview of genes whose gain or loss-of-function alters contraction/exercise-stimulated glucose uptake. Indicated in table is the study (author), manipulated gene, whether manipulation was gain or loss-of-function, average glucose uptake (of all measured muscles) and effect on muscle mass.

Author	***Gene***	**GAIN or LOSS**	**Average difference glucose uptake**	**Effect on muscle mass**
(Ayala et al., 2009)	*Glp1r*	LOSS	80%	Not specified
(Kang et al., 2012)	*Mcat*	GAIN	75%	Not specified
(Kang et al., 2012)	*Sod2*	GAIN	60%	Not specified
(Yu et al., 2015)	*Prkcd*	LOSS	35%	No effect
(Liu et al., 2007)	*Dgat1*	GAIN	30%	Not specified
(Jorgensen et al., 2004)	*Prkaa2*	LOSS	25%	Not specified
(Toyoda et al., 2011)	*Myo1c*	GAIN	20%	Not specified
(Liu et al., 2020)	*Tlr9*	LOSS	-20%	Not specified
(Jorgensen et al., 2004)	*Prkaa1*	LOSS	-25%	Not specified
(Sylow et al., 2013a)	*Rac1*	LOSS	-34%	Not specified
(He et al., 2012)	*Bcl2*	LOSS	-35%	No effect
(Benrick et al., 2012)	*Il6*	LOSS	-40%	Not specified
(Szekeres et al., 2012)	*Tbc1d1*	LOSS	-44%	No effect
(He et al., 2012)	*Becn1*	LOSS	-45%	Not specified
(Henríquez-Olguin et al., 2019)	*Ncf1*	LOSS	-53%	No effect
(Sakamoto et al., 2005)	*Stk11*	LOSS	-63%	No effect
(Tang et al., 2017)	*Chga*	LOSS	-65%	Decrease

Eidesstattliche Erklärung

Anhang I

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die bei der promotionsführenden Einrichtung
Fakultät für Sport- und Gesundheitswissenschaften

der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:
On the interplay between glucose metabolism and skeletal muscle hypertrophy

in der Fakultät für Sport- und Gesundheitswissenschaften, Professur für Sportbiologie
Fakultät, Institut, Lehrstuhl, Klinik, Krankenhaus, Abteilung

unter der Anleitung und Betreuung durch: Prof. Dr. Henning Wackerhage ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Ab. 6 und 7 Satz 2 angebotenen Hilfsmittel benutzt habe.

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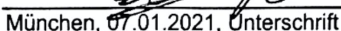

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