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Defining the contribution of the protein deacetylase, sirtuin 1, to contraction-stimulated glucose uptake in mice

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by

Ji Hyun Kang

Committee in charge:

Professor Simon Schenk, Chair
Professor Randolph Hampton, Co-Chair
Professor Kimberly Cooper

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The Thesis of Ji Hyun Kang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2020

DEDICATION

I dedicate this to my parents whose sacrifices and love allowed me to accomplish all that I aspire and become the person I am today. Thank you for everything.

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ABBREVIATIONS

ATP	Adenosine triphosphate
GLUT4	Glucose transporter 4
AMPK	AMP-activated protein kinase
AMP	Adenosine monophosphate
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
TBC1D1	TBC1 domain family member 1
EDL	Extensor digitorum longus
PTM	Post-translational modification
KAT	acetyltransferase
DAC	deacetylase
EXOC	Exocyst complex component
MYO	Myosin
Rab	Ras-related proteins
RhoGDIα	Rho guanine nucleotide dissociation inhibitor α
TUG	Tether containing a UBX domain of GLUT4
VAMP	Vesicle-associated membrane protein
VAP	VAMP-association protein
NAD^+	Nicotinamide adenine dinucleotide
SIRT	Sirtuin
SIRT1	Sirtuin 1
FOXO	Forkhead box transcription factors protein
PGC-1α	Proliferator-activated receptor gamma coactivator 1-alpha

LKB	Liver kinase B
KHB	Krebs-Henseleit buffer
2DOGU	2-deoxyglucose uptake
HKII	Hexokinase II

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ABSTRACT OF THE THESIS

Defining the contribution of the protein deacetylase, sirtuin 1, to contraction-stimulated glucose uptake in mice

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Ji Hyun Kang

Master of Science in Biology

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Professor Simon Schenk, Chair
Professor Randolph Hampton, Co-Chair

Exercise (i.e. contraction) robustly stimulates skeletal muscle glucose uptake, making it a cornerstone non-pharmaceutical intervention for treating clinical hyperglycemia and insulin-resistance. Remarkably, however, the molecular steps that regulate contraction-stimulated glucose uptake by skeletal muscle remain incompletely defined. The mammalian ortholog of Sir2, sirtuin 1 (SIRT1), is a protein deacetylase that is thought to link perturbations in energy flux associated with exercise, particularly at the level of NAD^+ and NAD^+/NADH, to subsequent cellular adaptations. Nevertheless, its role in contraction-stimulated glucose uptake has not been defined.

The objective of this study was to determine the contribution of SIRT1 to contraction-stimulated glucose uptake in mouse skeletal muscle. Using radioactive 2-deoxyglucose uptake (2DOGU) approach, we measured *ex vivo* glucose uptake in unstimulated and electrically-stimulated (100 Hz contraction [2s train, 0.2ms pulse, 35V] every 15s for 10 min) extensor digitorum longus (EDL) and soleus from ~15 week old male and female mice with muscle-specific knockout of SIRT1 (mKO) and their wildtype (WT) littermates. As expected, force output decreased over the contraction protocol, although there were no differences in the rate of fatigue between genotypes. In EDL and soleus from WT mice, 2DOGU was ~2.5-fold higher in contracted vs. rested muscle, regardless of sex, and this effect was not impaired in mKO mice. Interestingly, the absolute rate of contraction-stimulated 2DOGU was ~1.3-fold higher in both the soleus and EDL of female compared to male mice, regardless of genotype. Taken together, our findings demonstrate that SIRT1 is not required for contraction-stimulated glucose uptake in mouse skeletal muscle.

INTRODUCTION

1.1 Beneficial effects of exercise on health and glucose homeostasis

Type 2 diabetes is a prevalent and complex disease that affects 31 million people in the United States alone (CDC, 2020). A primary hallmark of type 2 diabetes is fasting and post-prandial hyperglycemia and insulin resistance, particularly in skeletal muscle (DeFronzo & Tripathy, 2009). This latter point is important, as skeletal muscle is responsible for as much as ~85% of peripheral glucose uptake after a meal (DeFronzo *et al.*, 1981, 1985; James *et al.*, 1985; Kraegen *et al.*, 1985; Yki-Jarvinen *et al.*, 1987; Bonadonna *et al.*, 1993). Thus, the physiological action of insulin, particularly in the skeletal muscle, is fundamental to health. Impaired skeletal muscle insulin sensitivity underlies the etiology of type 2 diabetes (Lillioja *et al.*, 1988, 1993; Weyer *et al.*, 1999; Facchini *et al.*, 2001) and predicts disease risk (Facchini *et al.*, 2001). Moreover, type 2 diabetes is characterized by extensive financial (Hogan *et al.*, 2003; Huang *et al.*, 2009; Zhuo *et al.*, 2013) and personal burden (Hogan *et al.*, 2003; Johannesson *et al.*, 2009; Vamos *et al.*, 2010; Yau *et al.*, 2012; Fathy *et al.*, 2016). Physical activity is a cornerstone intervention for the prevention and treatment of type 2 diabetes (Zinman & Vranic, 1985; Wing *et al.*, 1988; Eriksson & Lindgärde, 1991; Ivy *et al.*, 1999; Henriksen, 2001; Stanford & Goodyear, 2014). A major reason for this is because skeletal muscle contraction (i.e. exercise) increases the clearance of glucose from the blood independent of insulin (Gao *et al.*, 1994; Lund *et al.*, 1995; Ivy *et al.*, 1999; Messina *et al.*, 2015; Colberg *et al.*, 2016), and as such, this beneficial effect of exercise is preserved in insulin-resistant muscle (Wallberg-Henriksson & Holloszy, 1984; Richter *et al.*, 1985; McConell *et al.*, 1994; Kennedy *et al.*, 1999; Kusunoki *et al.*,

2010). Therefore, even in the context of insulin resistance, exercise is a highly effective and implementable non-pharmacological approach to improve overall health and reduce secondary health complications arising from hyperglycemia and type 2 diabetes.

Contraction and glucose uptake. Adenosine triphosphate (ATP) is a fundamental energy currency that is used by skeletal muscle to generate force (Hargreaves, 2000; Hargreaves & Spriet, 2018); for that reason, maintenance of ATP availability during contraction is critical to maintaining force production (Robergs *et al.*, 2004; Caruel & Truskinovsky, 2018). Glucose is metabolized by skeletal muscle to generate ATP, especially with increasing exercise intensity (Romijn *et al.*, 1993; Coyle, 1995, 2000; Mul *et al.*, 2015). For example, Romoijn *et al.* demonstrated that when increasing intensity from 25 to 65 and then to 85% of maximal oxygen consumption (VO_{2max}), there is a stark transition from fat to carbohydrate (i.e. glucose) as the primary source of ATP production in endurance-trained humans (Romijn *et al.*, 1993). There are two primary sources of glucose that are used by the contracting muscle: glucose that is released from muscle glycogen and glucose that is taken up by muscle from the blood (Romijn *et al.*, 1993, 2000; Hargreaves, 2015; Hargreaves & Spriet, 2020). The first experiment demonstrating that contraction stimulates ("external") glucose uptake was performed in frog muscle in 1965 by John O. Holloszy where he observed an ~20-fold increase in glucose uptake compared to basal with tetanic stimulation (Holloszy & Narahara, 1965). Following this discovery, many studies over the last ~55 years have demonstrated that contraction/exercise increases skeletal muscle glucose uptake in humans (McConell *et al.*, 1994; Kennedy *et al.*, 1999) and rodent muscles (Ivy & Holloszy, 1981; Richter *et al.*, 1985; Sylow *et al.*, 2017).

Contraction and plasma membrane GLUT4. The uptake of glucose by the skeletal muscle is regulated by the facilitated diffusion of glucose via glucose transporter 4 (GLUT4; also known as SLC2A4 gene). GLUT4 is the most abundant isoform of glucose transporters expressed in skeletal muscle and resides both in intracellular GLUT4 storage vesicles and at the plasma membrane (Richter *et al.*, 2003; Evans *et al.*, 2019). At rest, glucose uptake by the muscle is low with GLUT4 primarily being intracellular (i.e. away from the plasma membrane) (Wojtaszewski & Richter, 1998). Contraction can increase glucose uptake by as much as ~40-fold in human muscle (Richter & Hargreaves, 2013), with this increase being dependent on the translocation of the GLUT4 from its intracellular pool to the plasma membrane and t-tubules. This was first demonstrated by studies ~30 years ago that found an increase in GLUT4 in the plasma membrane fraction in response to exercise (Ezaki *et al.*, 1992; Sherman *et al.*, 1993; Ren *et al.*, 1994; Roy & Marette, 1996; Kristiansen *et al.*, 1996) and the amount of GLUT4 at the plasma membrane strongly correlated with the degree of glucose uptake. Since then, the obligatory role of GLUT4 for glucose uptake into skeletal muscle has been demonstrated in GLUT4 knockout mice where glucose uptake in response to electrical stimulation and *in vivo* exercise is completely abrogated (Fueger *et al.*, 2007; Howlett *et al.*, 2013; Richter & Hargreaves, 2013).

Summary. It is clear that exercise stimulates glucose uptake into skeletal muscle, regardless of its responsiveness to insulin, and it does so via the translocation of GLUT4 to the plasma membrane. In the next section, I will discuss potential signaling pathways and changes that underlie the regulation of contraction-stimulated GLUT4 translocation and glucose uptake.

1.2 Potential molecular mediators of contraction-stimulated GLUT4 translocation and glucose uptake in skeletal muscle

Before an overview of potential molecular regulators of contraction-stimulated glucose uptake, it is important to note that mechanical load and exercise-induced increases in cytosolic calcium are also possible signals that promote, at least in part, GLUT4 translocation to the plasma membrane and glucose uptake in skeletal muscle. However, these mechanisms of regulation are outside the focus of this Thesis; readers can find some excellent papers and comprehensive reviews on these topics elsewhere (Richter *et al.*, 2001, 2003; Rose & Richter, 2005; Richter & Hargreaves, 2013).

AMP (adenosine monophosphate)-activated protein kinase (AMPK). The AMP/ATP ratio, and AMP in particular, increases during exercise in an intensity-dependent manner (Wilson, 1995; Dash *et al.*, 2008; Herzig & Shaw, 2018). AMPK is an evolutionary conserved heterotrimeric (αβγ) kinase that is allosterically activated by an increase in AMP concentration. When AMPK is activated, ATP consuming processes (e.g. fatty acid, cholesterol, and glycogen synthesis) are inhibited and ATP generating pathways (e.g. fatty oxidation, glycolysis, and glucose uptake) are activated (Jørgensen *et al.*, 2006; O'Neill, 2013; Lantier *et al.*, 2014; Garcia & Shaw, 2018). Therefore, due to its sensitivity to energetic stress, AMPK has long been thought to be a key modulator that connects the metabolic stress of exercise to an increase in muscle glucose uptake (Jørgensen *et al.*, 2006; Jensen *et al.*, 2009; Kjøbsted *et al.*, 2019). Early *in vivo* studies in which skeletal muscle AMPK activity in rats increased during exercise were the reason AMPK was thought to be a master regulator (Winder & Hardie, 1996). Additionally, pharmacological activation of AMPK by 5-aminoimidazole-4-

carboxamide ribonucleoside (AICAR) increased skeletal muscle glucose uptake and free fatty acid oxidation in rat hindlimbs (Merrill *et al.*, 1997). AMPK also has a glycogen binding domain, so it is thought to be sensitive to changing glycogen concentration during exercise (McBride *et al.*, 2009), which itself can be a major determinant of skeletal muscle glucose uptake during exercise (Bergström *et al.*, 1967; Blomstrand & Saltin, 1999; Steensberg *et al.*, 2002; Hargreaves, 2015) As a result of these studies, and others (Lefort *et al.*, 2008; Cantó *et al.*, 2013; Jensen *et al.*, 2014; Pataky *et al.*, 2020; Yue *et al.*, 2020), AMPK was implicated as an important mediator of exercise and glucose uptake.

A number of studies corroborated early findings that AMPK was important for exercise stimulated glucose (Jørgensen *et al.*, 2006; Lefort *et al.*, 2008; Sylow *et al.*, 2017). However, more recent studies have contested an obligatory role for AMPK (Kjøbsted *et al.*, 2019; Hingst *et al.*, 2020; McConell, 2020). A potential reason for the discrepancies is that current evidence for AMPK-mediated regulation of glucose uptake during exercise are predominantly based on transgenic model with lifelong (chronic) impairments of AMPK function. This approach introduces bias to previous findings due to the confounding variables and secondary effects resulting from chronic disruptions to AMPK function. In fact, recent studies utilizing an inducible muscle-specific AMPK-deficient mice (i.e. temporal ablation of both AMPK α1 and α2) have clearly demonstrated that glucose uptake during *ex vivo* and *in situ* contractions is not impaired during contraction, but they are required for glucose uptake post-exercise/recovery (Kjøbsted *et al.*, 2019). An inducible AMPK knockout model analogously suggests that AMPK is critical for nucleotide balance but not necessary for muscle glucose uptake

and fat oxidation during *in vivo* treadmill exercise (Hingst *et al.*, 2020). Taken together, recent studies demonstrate that AMPK is a minor contributor to skeletal muscle glucose uptake during contraction/exercise.

TBC1 domain family member 1 (TBC1D1). TBC1D1 is a Rab-GTPase (Rab-Gab) activating protein (GAP) that is closely related to AS160 (TBC1D4). TBC1D1 is highly expressed in the skeletal muscle and is thought to be a potential mediator of exercise- and contraction-stimulated glucose uptake. Rab-Gab proteins are important for inhibiting GLUT4 translocation in the absence of external stimuli by maintaining Rab proteins in the inactive (GDP-bound) state (Cartee, 2015). Several studies have demonstrated exercise/contraction to increase TBC1D1 phosphorylation in human (Jessen *et al.*, 2011; Treebak *et al.*, 2014) and rodent muscles (An *et al.*, 2010) and importantly, the phosphorylation of TBC1D1 promotes GLUT4 translocation by activating the Rab proteins associated with the formation, transport, tethering, and fusion of vesicles (Hook *et al.*, 2020). The role of TBC1D1 in contraction-stimulated glucose uptake is supported by TBC1D1 transgenic rodent models. For instance, mice with whole-body knockout of TBC1D1 exhibited a decrease in glucose uptake in the extensor digitorum longus (EDL) with ~50% reduction during *in vivo* exercise and ~85% reduction during in *in situ* contraction (Kjøbsted *et al.*, 2019). Additionally, Stöckli *et al.* demonstrated that the loss of whole-body TBC1D1 in mice resulted in a ~30% reduction of *in vivo*-mediated glucose uptake in the white quadriceps, however this effect was not observed in the red quadriceps (Stöckli *et al.*, 2015). Importantly, the loss of TBC1D1 resulted in a decreased GLUT4 protein abundance in the fast-twitch muscles (white quadriceps, tibialis anterior, and EDL) with no differences observed in

slow-twitch muscles (soleus and red quadricep) (Stöckli *et al.*, 2015). Together, these findings suggest a fiber-type specific role of TBC1D1 in the recruitment of GLUT4 and contraction-stimulated glucose uptake in the skeletal muscle, although this effect could be, at least in part, due to reduction in GLUT4 abundance.

Rac1 (Rac Family Small GTPase 1). Rac1 is a member of the Rac subfamily of the family Rho family of GTPases and regulates diverse cellular processes including cell growth, activation of protein kinases, NADPH oxidase-dependent reactive oxygen species production, and remodeling of the actin cytoskeleton. Rac1 is activated by both exercise and passive stretch, and as such, has been proposed to be important to contraction-stimulated glucose uptake (Sylow *et al.*, 2013, 2014, 2016; Yue *et al.*, 2020). To this point, in mice with inducible muscle-specific knockout of Rac1, *ex vivo* contraction-stimulated glucose uptake was impaired by ~20% in soleus and EDL compared to WT (Sylow *et al.*, 2013). Similarly, exercise-stimulated glucose uptake was reduced in the Rac1 muscle knock-out mice during *in vivo* exercise which was positively correlated with a ~25% reduction in plasma membrane GLUT4 compared to WT mice (Sylow *et al.*, 2016). Despite the number of studies suggesting Rac1 to be critical for contraction-stimulated glucose uptake, the loss of Rac1 only resulted in a modest decrease in contraction-stimulated glucose uptake and GLUT4 abundance at the plasma membrane. As such, while Rac1 might contribute to contraction-stimulated glucose uptake, it is certainly is a minor contributor.

Summary. AMPK, TBC1D1, and Rac1 have been proposed as key points of control for contraction-stimulated glucose uptake. However, recent studies in AMPK transgenic mice have robustly demonstrated that AMPK is not required for increased

glucose uptake *during* contraction. Pharmacological and transgenic ablation of TBC1D1 and Rac1 have shown minimal to modest reductions in exercise- and contraction-stimulation glucose, and these effects are also dependent on muscle fiber type (Sylow *et al.*, 2013, 2017; Stöckli *et al.*, 2015; Kjøbsted *et al.*, 2019). Indeed, even combined ablation of both AMPK and Rac1 does not fully block contraction-stimulated glucose uptake in mouse skeletal muscle (Sylow *et al.*, 2017). Thus, it is clear that hitherto unknown molecular signals (and proteins) must be fundamental to contraction-stimulated glucose uptake in the skeletal muscle.

1.3 Acetylation and contraction-stimulated glucose uptake

Basics of lysine acetylation. Post-translational modifications (PTMs) are chemical changes to a protein that leads to altered functionality, be it through changes in activity, localization, and/or ability to interact with other molecules (Philp *et al.*, 2014). Examples of PTMs include phosphorylation, lysine acetylation, methylation, glycosylation, and ubiquitination (Knorre *et al.*, 2009; Duan & Walther, 2015). Lysine acetylation (or simply, "acetylation"), which is the focus of this Thesis, is a reversible and dynamic modification of a lysine residue (or residues) on a protein. At the enzymatic level, lysine acetylation is balanced by the activity of acetyltransferases and deacetylases, which attach and remove acetyl groups from lysine residues, respectively (Sterner & Berger, 2000) (Figure 1). Acetylation was initially discovered over 50 years ago as a regulator of transcription via acetylation of histone proteins by its effects on RNA synthesis rate (Verdin & Ott, 2015). More recently, the importance of protein acetylation has expanded well beyond transcription to virtually all aspects of cellular

homeostasis, including chromatin remodeling, cell cycle control, splicing, metabolism, cell growth and nuclear transport, to name just a few. In fact, proteomic studies reveal that lysine acetylation is as common in cells as phosphorylation (Polevoda & Sherman, 2002; Knorre *et al.*, 2009; Zhao *et al.*, 2011).

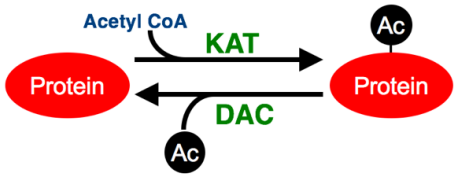

Figure 1. Lysine acetylation is regulated by the activity of acetyltransferases (KAT) and deacetylases (DAC), which add and remove acetyl groups, respectively.

Acetylation and GLUT4 trafficking proteins. GLUT4 trafficking is mediated by a series of molecular signals that result in the movement of GLUT4 from its intracellular compartment to the plasma membrane. Although a detailed overview of this process is beyond the scope of this Thesis, there are many excellent reviews that cover this topic (Richter *et al.*, 2004; Jessen & Goodyear, 2005; Richter & Hargreaves, 2013; Klip *et al.*, 2019). Briefly, the 5 steps of GLUT4 translocation are: *1)* retention/release; *2)* transport; *3)* microtubule and actin remodeling; *4)* tethering; *5)* docking and fusion. Interestingly, robust discovery-based (and unbiased) proteomic studies that specifically measured lysine acetylation have identified integral proteins at all steps of GLUT4 trafficking (Kim *et al.*, 2006; Choudhary *et al.*, 2009; Wang *et al.*, 2010; Zhao *et al.*, 2011; Lundby *et al.*, 2012; Zencheck *et al.*, 2012; Belman *et al.*, 2015; LaBarge *et al.*, 2015; Aslan *et al.*, 2015; Weinert *et al.*, 2018) (See, Table 1). While lysine acetylation of this proteins is interesting, whether their acetylation is

necessary for contraction-stimulated GLUT4 movement to the plasma membrane and subsequent glucose uptake is unknown, as is the identity of the acetyltransferase(s) and deacetylase(s) that regulate their acetylation.

Table 1. Examples of GLUT4 trafficking proteins with an acetylated lysine residue	
Step of GLUT4 trafficking	Protein
Retention & Release	TUG, Golgin-160
Transport	KIF5B, MYO1C, MYO5A, Rab8A, Rab14
Microtubule & actin reorganization	RhoGDIα, p21-activated kinase, filamin C
Tethering	EXOC 1-4 & 8, syntaxin 4
Docking and Fusion	VAMP3, VAPA
EXOC, exocyst complex component; MYO, myosin; Rab, ras-related proteins; RhoGDIα, Rho guanine nucleotide dissociation inhibitor α; TUG, tether containing a UBX domain for GLUT4; VAMP, vesicle-associated membrane protein; VAP, VAMP-associated protein	

Acetyl CoA and NAD$^+$ (nicotinamide adenine dinucleotide): Linking exercise to lysine acetylation. Acetyl-CoA and NAD$^+$ are fundamental metabolic intermediates across all aspects of cellular substrate metabolism, including in skeletal muscle during exercise. For example, the flux and concentrations of these metabolites is robustly perturbed by exercise intensity and duration, both in the cytosol and mitochondria (Dash *et al.*, 2008; Li *et al.*, 2009; White & Schenk, 2012). This is of potentially high relevance to this Thesis, as acetyl-CoA are also intimately linked to the regulation of lysine acetylation. To this point, acetyl-CoA is the substrate of acetyltransferases, whilst NAD$^+$ is the substrate of some members of the sirtuin (SIRT) family of deacetylases. Thus, it is possible that perturbations in cellular acetyl-CoA and/or NAD$^+$ during exercise causally link the metabolic stress of exercise to a contraction-stimulated increase in GLUT4 translocation to the plasma membrane and glucose uptake. Exercise increases NAD$^+$ and NAD$^+$/NADH ratio in skeletal muscle (Wendt & Chapman, 1976;

Duboc *et al.*, 1988; Czubryt *et al.*, 2003; Cantó *et al.*, 2010; Koltai *et al.*, 2010; White & Schenk, 2012), although this finding is not universal (Hansford, 1975; Graham *et al.*, 1976; Schiøtz Thorud *et al.*, 2004; Cantó *et al.*, 2013). Specific to this Thesis, I will be focusing on the potential contribution of deacetylation, and in particular, the protein deacetylase, sirtuin 1 (SIRT1), to the regulation of contraction-stimulated glucose uptake.

SIRT1. The SIRT family of proteins are a highly conserved family of class III NAD^+ dependent deacetylases. Seven mammalian SIRTs (SIRT 1-7) have been identified; they are all ubiquitously expressed and share a common catalytic core domain but each SIRT has a distinct subcellular location and substrate specificity that helps differentiate their various functions (Dali-Youcef *et al.*, 2007). While the focus of this Thesis is on SIRT1, more encompassing reviews on SIRT family and their functions can be found elsewhere (Dali-Youcef *et al.*, 2007; Pardo & Boriek, 2011; Rahman & Islam, 2011; Kelsey C. Martin Mhatre V. Ho, 2012; Vargas-Ortiz *et al.*, 2019). SIRT1 is a mammalian ortholog of the yeast silent information regulator 2 (sir2). It was first discovered to be a longevity protein that increased life span in yeast (Defossez *et al.*, 2001). Since then, the field of SIRT1 biology has exploded, with SIRT1 being implicated in numerous aspects of cellular biology and health, including, metabolism, cellular stress, longevity, and cancer. A major reason for this is that SIRT1 deacetylates (and regulates) relevant proteins, such as forkhead box transcription factors proteins (FOXO), p53, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), nuclear factor kappa-light-chain-enhancer of activated B cells, sterol response element binding proteins, and liver X receptors (Rahman & Islam, 2011). As it

relates to this Thesis, though, why focus on SIRT1?

Exercise and SIRT1. Both chronic exercise training (Gurd *et al.*, 2010; Huang *et al.*, 2016; Chen *et al.*, 2018; Liu *et al.*, 2019) and an single session of exercise (Suwa *et al.*, 2008; Dumke *et al.*, 2009; Cantó *et al.*, 2010; Guerra *et al.*, 2010) increases SIRT1 activity in rodent and human skeletal muscle. For instance, 12-weeks of chronic swimming exercise training in rats increased SIRT1 protein by ~20% in the gastrocnemius and ~30% in the soleus compared to sedentary rats. Downstream targets of SIRT1 such as PGC-1α and FOXO3a (by 45% and 66% respectively) were also increased in the gastrocnemius and soleus following exercise training in rats (Huang *et al.*, 2016). Similarly, 6-weeks of high-intensity interval training in human muscle also increased SIRT1 activity by 31%, intrinsic activity per SIRT1 protein by 58%, and PGC-1α protein abundance by 16% compared to pre-training (Gurd *et al.*, 2010). Related to acute exercise, in humans, 3 hours of cycling increased SIRT1 mRNA levels by 2.5-fold (Dumke *et al.*, 2009), whilst a single bout of sprint exercise increased SIRT1 protein abundance (Guerra *et al.*, 2010). In rodent models, a single bout of treadmill running in rats increased SIRT1 and PGC-1α protein expression in the soleus (by 18% and 29%, respectively) (Suwa *et al.*, 2008) and 2.5 hours of swimming increased SIRT1 activity in mice (Cantó *et al.*, 2010). Thus, these findings, as well as others (Wright *et al.*, 2007; Rodgers *et al.*, 2008; Gibala *et al.*, 2009; Little *et al.*, 2010; Gurd *et al.*, 2011; White & Schenk, 2012; Menzies *et al.*, 2013; Chen *et al.*, 2018; Vargas-Ortiz *et al.*, 2019), suggest that both chronic and acute exercise activates SIRT1.

SIRT1 and AMPK. SIRT1 and AMPK are proposed to be an interdependent energy sensing network that contribute to exercise-induced adaptations (Cantó &

Auwerx, 2008, 2009; Cantó *et al.*, 2010). *In vitro* and *in vivo* findings demonstrate that AMPK is an upstream regulator of SIRT1 activity in skeletal muscle in response to fasting and exercise (Cantó & Auwerx, 2009; Cantó *et al.*, 2010, 2013). For instance, AICAR treatment of C2C12 myotubes decreased PGC-1α acetylation, an indication of increased SIRT1 activity, while the overexpression of AMPKα1 resulted in significant deacetylation of PGC-1α. Similarly, AICAR injections in mice results in decreased PGC-1α acetylation in the EDL and gastrocnemius. AMPK is thought to modulate SIRT1 activity by indirectly altering intracellular NAD^+/NADH ratio. To this point, AICAR treatment in C2C12 myotubes and mice skeletal muscle increased intracellular NAD^+/NADH ratio (Cantó *et al.*, 2013).

In contrast, other studies suggest that SIRT1 functions as an upstream regulator for AMPK signaling via modulating liver kinase B (LKB) (Hou *et al.*, 2008; Lan *et al.*, 2008; Suchankova *et al.*, 2009). LKB is a serine-threonine protein kinase that phosphorylates AMPK (Shackelford & Shaw, 2009). Lan et al. (2008) demonstrated that catalytically inactive SIRT1 in Hek293T cells increase LKB1 acetylation by 3-fold compared to control. Likewise, SIRT1 knockdown by shRNA increased LKB1 acetylation by 5.5-fold suggesting that SIRT has a direct role in modulating the acetylation status of LKB1 (Lan *et al.*, 2008). SIRT1, through its effect on LKB1, has been shown to further impact AMPK activity. For instance, pharmacological activation of SIRT1 by resveratrol increased the phosphorylation of AMPK in hepatic cells (Hou *et al.*, 2008; Suchankova *et al.*, 2009) and rat EDL muscle (Suchankova *et al.*, 2009) while inhibiting SIRT1 by nicotinamide decreased the phosphorylation of AMPK and its downstream target, acetyl-coA carboxylase (Hou *et al.*, 2008; Suchankova *et al.*,

2009). Taken together, despite the discrepancies in the primordial trigger, these studies suggest a unique signaling interdependence between AMPK and SIRT1.

Summary. Acetylation is crucial for various cellular processes and SIRT1 has numerous nuclear and non-nuclear targets. In fact, proteomic studies demonstrate that all steps of GLUT4 translocation involve cytosolic protein acetylation. By extension, SIRT1 which also exist in the cytosol may impact the acetylation status of GLUT 4 trafficking proteins. The rationale for investigating the role of SIRT1 is that exercise, both chronic and acute, increases SIRT1 activity and AMPK-SIRT1 interdependence suggest that there may be an important role of SIRT1 in regulating exercise-mediated changes in glucose uptake.

1.4 Gaps in knowledge

It has been over 50 years since the initial studies demonstrating that exercise/contraction increases skeletal muscle glucose uptake. As outlined above, while proteins such as AMPK, Rac1 and TBC1D1 may be sufficient as regulators of contraction-stimulated glucose uptake, they are not essential. This is demonstrated by the fact that their loss or inhibition only partially affects (or does not affect at all) contraction-stimulated glucose uptake. Additionally, their regulatory effect is dependent on muscle 'type'; in our hands, if a pathway is "fundamental" then it would impact contraction-stimulated glucose uptake regardless of fiber type. Thus, a clear gap in knowledge in the field is the identity of the signal(s) and intracellular signaling steps that regulate contraction-stimulated glucose uptake (and as part of this, contraction-stimulated GLUT4 translocation to the plasma membrane).

1.5 Research objective and hypothesis of this Thesis

The primary objective of this Thesis is to determine whether SIRT1 is necessary for contraction-stimulated glucose uptake in mouse skeletal muscle. To do this, we will study mice with muscle-specific knockout of SIRT1 (mKO) mice, which were generated previously by our laboratory using Cre-LoxP methodology (Philp *et al.*, 2011; Schenk *et al.*, 2011). In these male and female mKO mice and their Cre negative (i.e. "wildtype"/control) littermates, we will electrically stimulate the EDL and soleus muscles, *ex vivo*, and assess glucose uptake using a dual-radioactive tracer approach. My hypothesis is that contraction-stimulated glucose uptake will be impaired in mKO mice and that this effect will occur in both muscles, regardless of sex.

RESULTS

Contractile function and fatigability were not different between mKO and WT mice.

Initial maximal tetanic tension and tetanic tension during the contraction protocol was not different between genotypes in the EDL or soleus from male (**Figures 2A** and **2E**, respectively) or female mice (**Figure 2C** and **Figure 2G**, respectively). As calculated from the force-time data, the fatigue index, which represents the time taken for force to decrease to 40% of initial force, was not different between genotypes, in the EDL or soleus of male (**Figure 2B** and **Figure 2F**, respectively) or female (**Figure 2D** and **Figure 2H**, respectively) mice.

Contraction-stimulated glucose uptake is comparable in mKO and WT mice

In the EDL from WT and mKO mice, 2DOGU uptake was significantly higher in the contraction versus rested in male (**Figure 3A**) and female (**Figure 3B**) mice; no genotype differences in 2DOGU uptake were found within rest or contraction. Similarly, in the soleus from WT and mKO mice, 2DOGU uptake was significantly higher in the contraction versus rest in male (**Figure 3D**) and female (**Figure 3E**) mice; no genotype differences in 2DOGU uptake were found within rest or contraction. To investigate whether there were sex differences in contraction-stimulated (C-Stim) 2DOGU uptake (i.e. Contraction 2DOGU minus Rest 2DOGU), we collapsed the data for WT and mKO mice within each muscle and compared male versus female; we were able to do this as there was no genotype difference in the rest or contraction conditions. Interestingly, C-Stim 2DOGU uptake was significantly higher in both the EDL (**Figure**

3C) and soleus (**Figure 3F**) of female compared to male mice.

Phosphorylation of p38MAPK and AMPK is increased similarly in mKO and WT mice after contraction

In the EDL, phosphorylation of AMPK (Thr172) and p38 MAPK (Thr180/Tyr182) was significantly higher in contraction versus rest in both male (**Figure 4A and 4B**, respectively) and female (**Figure 4C and 4D**, respectively), regardless of genotype. Moreover, there was no sex difference in the phosphorylation of these proteins. Both the total abundance of p38 MAPK and AMPK, and basal phosphorylation of p38 MAPK and AMPK was not different by genotype, rest/contraction, or sex.

Hexokinase II (HKII) abundance was not different between male and female mice

For insight into why C-Stim 2DOGU uptake was different between sexes we assessed HKII abundance. For this, we collapsed the data for WT and mKO mice and for rested and contracted muscles, as there was no genotype difference nor changes with contraction stimulation. HKII abundance in the EDL (**Figure 5A**) and soleus (**Figure 5B**) were not different between the male and female mice.

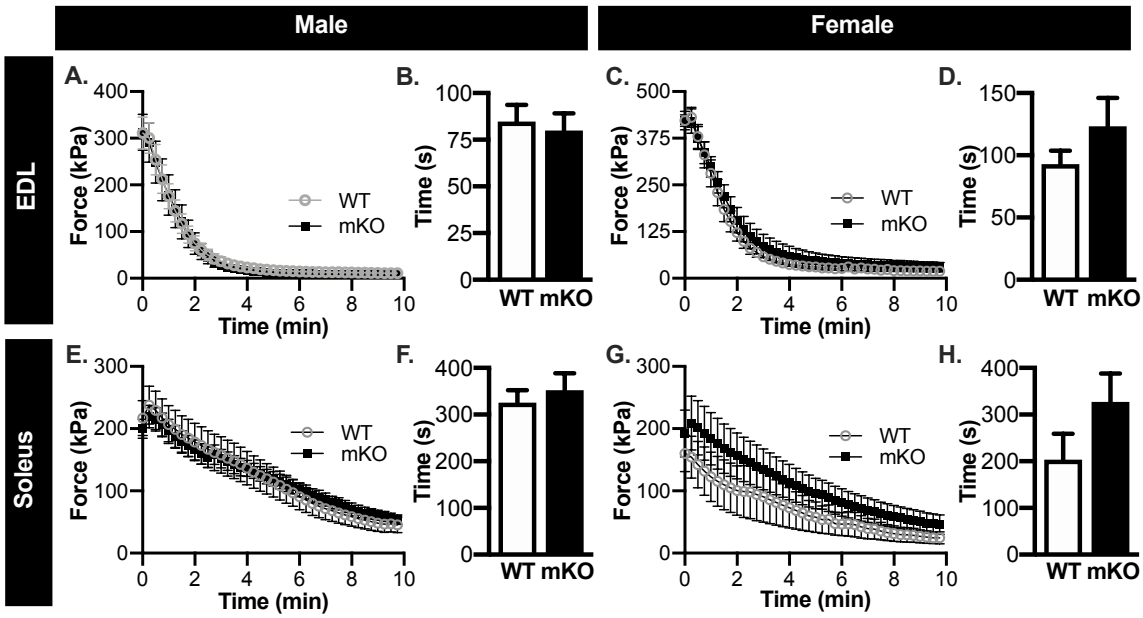

Figure 2. mKO mice show no impairments in contractile function or fatiguability compared to WT. In WT and mKO mice, temporal force production recording during 10-minute contraction protocol (40 subsequent contractions) in the EDL from ***(A)*** male and ***(C)*** female mice and soleus from ***(E)*** male and ***(G)*** female. In all muscles, there was a main effect of time (i.e. contraction) on force production, but no effect of genotype. Statistics: Data were analyzed by repeated measures two-way ANOVA. Fatigue index (time taken for force to decrease to 40% of initial force) in the EDL of ***(B)*** male and ***(D)*** female as well as soleus of ***(F)*** male and ***(H)*** female WT and mKO mice. Statistics: Data were analyzed by an unpaired t-test. Male: WT-Soleus, n=9; WT-EDL, n= 9; mKO-Soleus, n=7; mKO-EDL n= 7. Female: WT-Soleus, n=6; WT-EDL, n= 6; mKO-Soleus, n=6; mKO-EDL n=5. Data are presented as Mean±SEM.

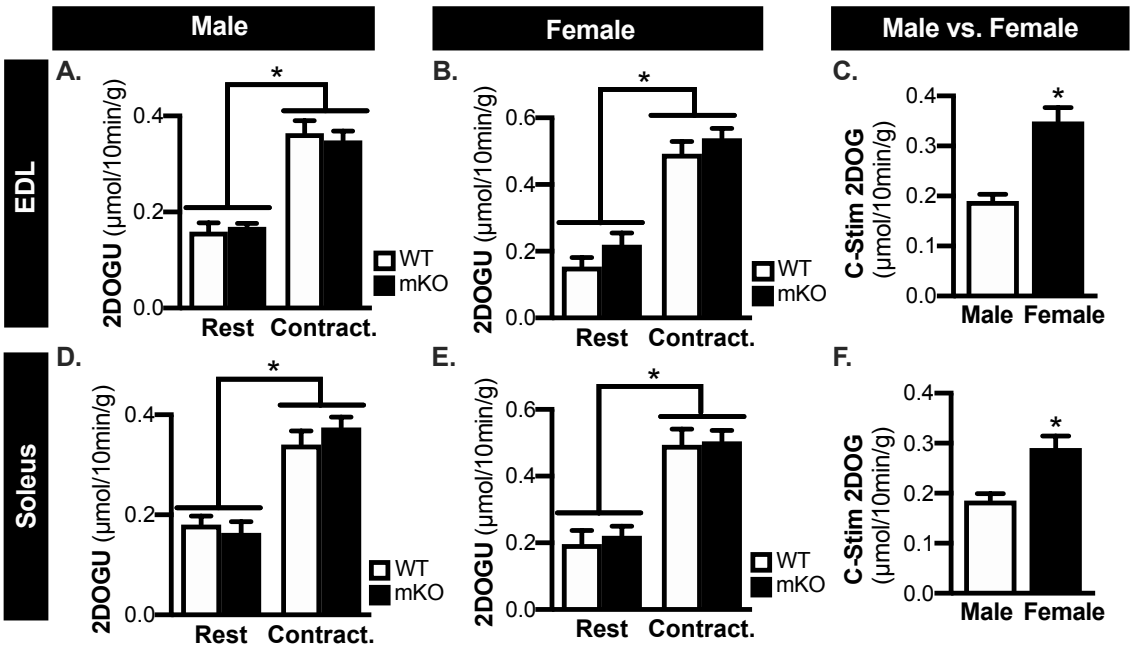

Figure 3. Contraction-stimulated glucose uptake is comparable in mKO and WT mice. In WT and mKO mice, rested (Rest) and contracted (Contract.) 2DOG uptake (2DOGU) in paired EDL from ***(A)*** male and ***(B)*** female, and paired soleus from ***(C)*** male and ***(D)*** female. There was a main effect of contraction on 2DOGU, but no effect of genotype. Statistics: Data were analyzed by a repeated measures two-way ANOVA. *P<0.05, main effect of contraction. Contraction-stimulated (C-Stim) 2DOGU (i.e., Contraction 2DOGU minus Rest 2DOGU) in the ***(C)*** EDL and ***(F)*** soleus from male and female mice; because there were no genotype differences in C-Stim 2DOGU data were collapsed together to assess sex differences. Statistics: Data were analyzed by an unpaired t-test. *P<0.05, versus Male. Male: WT-Soleus, n=7; WT-EDL, n= 9; mKO-Soleus, n=7; mKO-EDL, n= 7. Female: WT-Soleus, n=6; WT-EDL, n= 8; mKO-Soleus, n=7; mKO-EDL, n=8. Data are presented as Mean±SEM.

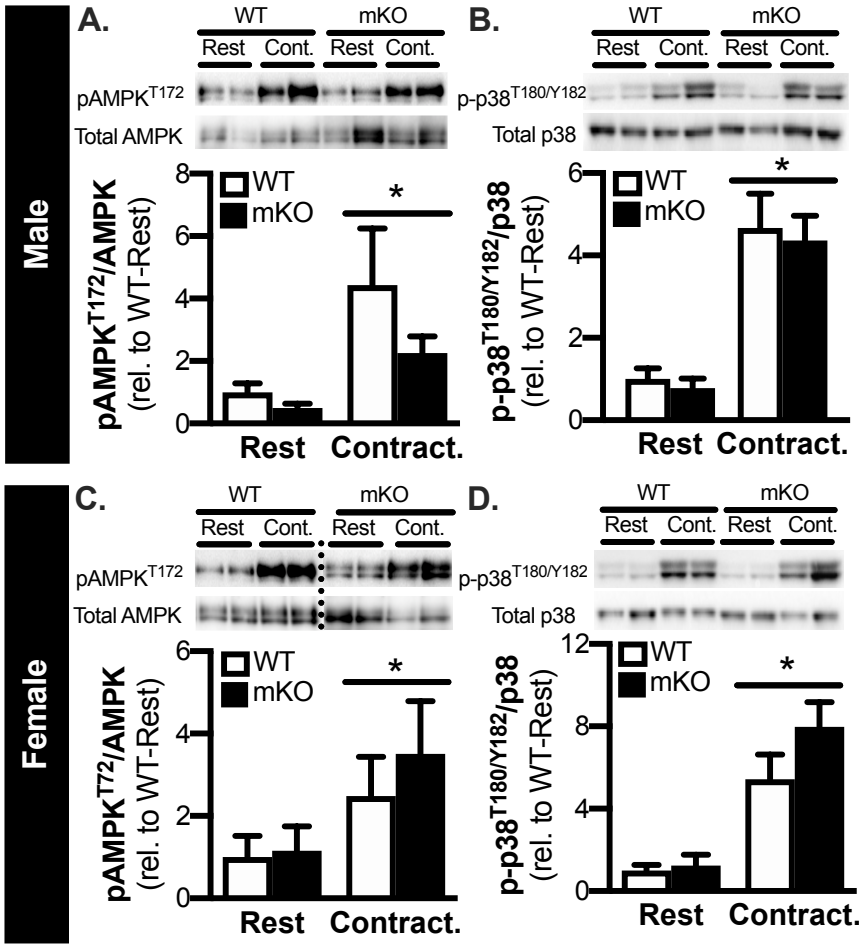

Figure 4. Contraction-stimulated increases in the phosphorylation of AMPK and p38 MAPK are similar in mKO and WT mice. Representative images and quantitation of ***(A & C)*** pAMPK^{T172} and ***(B & D)*** p-p38 MAPK$^{T180/Y182}$ from the rested (Rest) and contracted (Contract.) paired EDL from *(A-B)* male and *(C-D)* female, WT and mKO mice; analysis was conducted in the same muscles used for 2DOGU analysis. Protein phosphorylation was corrected for total abundance and the data are presented as relative (rel.) to WT-Rest. Dotted line indicates spliced images from gels that have been exposed together for the same duration of time. Statistics: Data were analyzed by a repeated measures two-way ANOVA. *P<0.05, main effect of contraction. Male: WT-AMPK, n=5; WT-p38 MAPK, n=6; mKO-AMPK, n=6; mKO-p38MAPK n=6. Female: WT-AMPK, n=5; WT-p38 MAPK, n=6; mKO-AMPK, n=5; mKO-p38MAPK n=5. Data are presented as Mean±SEM.

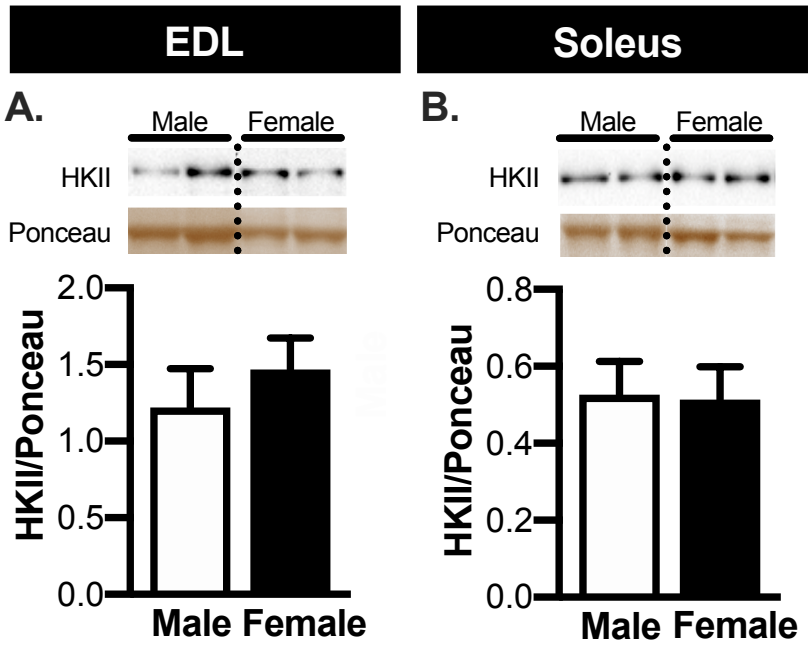

Figure 5. No difference in hexokinase II (HKII) abundance between male and female mice, regardless of muscle type. Representative images and quantitation of HKII (corrected for ponceau) in the **(A)** EDL and **(B)** soleus of male and female mice; because no effect of genotype or contraction was noted in HKII abundance within each sex, data were combined to test for sex differences in HKII abundance. Dotted line indicates spliced images from gels that have been exposed together for the same duration of time. Statistics: Data were analyzed by an unpaired t-test. Soleus: Male, n=8; Female, n=8. EDL: Male, n=13; Female, n=10. Data are presented as Mean±SEM.

DISCUSSION

The intracellular signals and signaling steps that link contraction to an increase in glucose uptake remain to be fully defined. Due to its sensitivity to changes in cellular NAD^+ concentration and the NAD^+/NADH ratio (Rahman & Islam, 2011; Cantó *et al.*, 2012, 2013; Cantó & Auwerx, 2013; Mouchiroud *et al.*, 2013), and inter-regulatory relationship with AMPK (Hou *et al.*, 2008; Lan *et al.*, 2008; Cantó & Auwerx, 2009; Suchankova *et al.*, 2009; Cantó *et al.*, 2010, 2013), SIRT1 has garnered attention as a potential integrator and effector of metabolic adaptations during exercise (Dali-Youcef *et al.*, 2007; Rodgers *et al.*, 2008; Gibala *et al.*, 2009; Banks A.L., Kon N., Knight C., Matsumoto M., Juarez R.G., Rossetti L., Gu W., 2011; Nogueiras *et al.*, 2012; Menzies *et al.*, 2013; Huang *et al.*, 2016; Chen *et al.*, 2018). Mechanistically, SIRT1 can regulate the activity/function of its protein substrates via deacetylation of lysine residues (Dali-Youcef *et al.*, 2007; Rahman & Islam, 2011). Indeed, SIRT1 has been linked to various aspects of muscle physiology and metabolism (Cantó & Auwerx, 2010; Erika Koltaia, Zsofia Szaboa, Mustafa Atalayb, Istvan Boldoghc, Hisashi Naitod, Sataro Gotoa,d, Csaba Nyakasa, and Zsolt Radaka, 2011; Gurd, 2011; White & Schenk, 2012; Vargas-Ortiz *et al.*, 2019; Radak *et al.*, 2020). Nevertheless, the importance of SIRT1 to contraction-stimulated glucose uptake has not been studied. To address this, we studied mice with muscle-specific knockout of SIRT1 deacetylase activity and measured glucose uptake in response to *ex vivo* electrical stimulation. Contrary to our hypothesis, our results demonstrate that the deacetylase function of SIRT1 is not necessary for contraction-stimulated glucose uptake.

Contraction/exercise robustly increases glucose uptake into skeletal muscle (Ren

et al., 1994; Lund *et al.*, 1995; Holloszy, 2003; Richter & Hargreaves, 2013), and for many years AMPK was considered a master regulator of this increase (Jørgensen *et al.*, 2006; Lefort *et al.*, 2008; Jensen *et al.*, 2009; Lantier *et al.*, 2014; Janzen *et al.*, 2018). While this regulation, at least in part, occurs through the AMPK-mediated phosphorylation and regulation of TBC1D1 (An *et al.*, 2010; Cartee, 2015; Stöckli *et al.*, 2015; Janzen *et al.*, 2018; Hook *et al.*, 2020), a number of studies have noted a signaling interplay and inter-dependence between SIRT1 and AMPK. For example, some studies suggest that AMPK is an important regulator of SIRT1 (via effects on NAD^+ and NAD^+/NADH ratio) (Cantó *et al.*, 2010, 2013), whilst other studies have proposed the opposite, such that SIRT1 as an important regulator of AMPK activity, through its ability to regulate liver kinase B1 (Hou *et al.*, 2008; Lan *et al.*, 2008; Suchankova *et al.*, 2009). Despite this purported interplay between AMPK and SIRT1, we find that loss of SIRT1 deacetylase activity does not impair either contraction-stimulated glucose uptake or AMPK phosphorylation/activation. This is in line with previous work from our lab demonstrating that AMPK phosphorylation and/or activity is not impacted by modulation of skeletal muscle SIRT1 activity (Philp *et al.*, 2011; Schenk *et al.*, 2011; White *et al.*, 2013; Svensson *et al.*, 2017), nor AICAR-stimulated glucose uptake impaired in SIRT1 muscle-specific knockout mice (Schenk *et al.*, 2011). It is also in line with a collection of studies demonstrating that AMPK is not required for contraction-stimulated glucose uptake (Kjøbsted *et al.*, 2019; Hingst *et al.*, 2020; McConell, 2020). Taken together, these results demonstrate that contraction-stimulated glucose uptake does not require SIRT1 (or AMPK).

SIRT1 has long been considered as an important regulator of mitochondrial

biogenesis in skeletal muscle, and by extension, the fatigability of skeletal muscle (Rodgers *et al.*, 2008; Pardo & Boriek, 2011). For instance, several studies have demonstrated that pharmacological activation of SIRT1, or activation of SIRT1 via elevation of cellular NAD^+ concentration and/or NAD^+/NADH ratio, increases skeletal muscle mitochondrial biogenesis (Bai *et al.*, 2012; Cantó *et al.*, 2012; Cantó & Auwerx, 2013; Mouchiroud *et al.*, 2013; Andreux *et al.*, 2014; Cerutti *et al.*, 2014; Houtkooper *et al.*, 2016; Vargas-Ortiz *et al.*, 2019). Similarly, in mice with a whole-body overexpression of SIRT1 (including in skeletal muscle), mitochondrial abundance and function are increased in skeletal muscle (Banks *et al.*, 2008). Additionally, in mice with muscle-specific overexpression of SIRT1, the gene and/or protein expression of some electron transport chain proteins and glycolytic and -oxidation enzymes are increased (Price *et al.*, 2012; White *et al.*, 2014). Interestingly though, such changes did not result in functional changes in mitochondrial respiration (Svensson *et al.*, 2017), time to fatigue during treadmill running, or fatigability in response to repeated electrical stimulation (Svensson *et al.*, 2020). In line with this, and our previous work in the EDL of mKO mouse (Philp *et al.*, 2011), we found that maximal tetanic tension and fatigability of both the soleus and EDL were not impacted in mKO mice, regardless of sex. Thus, when combined with other work from our lab (Philp *et al.*, 2011; White *et al.*, 2014; Svensson *et al.*, 2017, 2020) and others (Myers *et al.*, 2019), it is clear that SIRT1 is not a major regulator of skeletal muscle contractile function or fatigability.

To our knowledge, this is the first study to investigate the role of sex in the regulation of contraction-stimulated glucose uptake. Interestingly, we found that in the contracted muscle (but not rested), glucose uptake was ~30% higher in females versus

males in both the EDL and soleus. Considering the well-known difference in myosin heavy chain composition (i.e. fiber type) between these muscles in mice (Burkholder *et al.*, 1994; Augusto *et al.*, 2004), this suggests that this sex-based difference is perhaps "intrinsic". Providing support for this finding, Campbell and Febbraio (2002) found that contraction-stimulated glucose uptake by skeletal muscle during treadmill running was significantly lower (~50% reduction in red- and ~30% reduction in white quadricep) in estrogen-deficient female rats compared to the female controls (Campbell & Febbraio, 2002). However, this study did not compare male versus female rats. Nonetheless, this effect of contraction on glucose uptake is in line with numerous studies in rodent and human skeletal muscle demonstrating that insulin-stimulated glucose uptake and insulin sensitivity in skeletal muscle is higher in females compared to the males (Yki-Järvinen, 1984; Faure *et al.*, 1992; Nuutila *et al.*, 1995; Fernández-Real *et al.*, 1998; Tsao *et al.*, 2001; Hevener *et al.*, 2002; Horton *et al.*, 2006; Pfluger *et al.*, 2008; Lundsgaard & Kiens, 2014; Rattanavichit *et al.*, 2016; Tramunt *et al.*, 2020).

Skeletal muscle glucose uptake has three primary points of control: delivery, transport and metabolism of glucose in the cell (Richter *et al.*, 2004; Richter & Hargreaves, 2013). Because we used an ex vivo contraction approach, delivery is controlled, and as such, in our model the steps at which glucose uptake might be differently regulated between female and male mice could be transport, which is regulated by GLUT4 (Ryder *et al.*, 2001; Zorzano *et al.*, 2005; Richter & Hargreaves, 2013), and/or intracellular glucose metabolism, which is primarily (at least initially) regulated by glucose phosphorylation by HKII. While we did not assess GLUT4 abundance in this study, previous studies demonstrate that skeletal muscle GLUT4

protein abundance is not different in male versus female skeletal muscle (Houmard *et al.*, 1995; Høeg *et al.*, 2009) or in ovariectomized rats (Campbell & Febbraio, 2002), which suggests that differences in GLUT4 does not underlie the effects we see on contraction-stimulated glucose uptake. Alternatively, Høeg et al. demonstrated that HKII protein abundance is 56% higher in females compared to males (Høeg *et al.*, 2011). However, we found no sex difference in HKII abundance in this study. It should be noted that glucose uptake related to the amount of GLUT4 at the plasma membrane and not necessarily that total abundance of GLUT4. As such, it is possible that in female muscle that the dynamics of GLUT4 retention/release, translocation, docking, and fusion (independent of GLUT4 abundance) are differentially regulated compared to males, and it will be interesting in future work to study these areas.

It is important to discuss potential limitations of this work. The contraction protocol that we used was only for 10 minutes (and 40 total contractions). While this protocol clearly fatigued the soleus and EDL, it is possible that SIRT1 is important for contraction-stimulated glucose uptake during longer duration exercise (e.g. exercise that is hours in duration, rather than minutes). Moreover, we only measured *ex vivo* contraction-stimulated glucose uptake, which is an approach commonly used in the field because it allows tight control of the surrounding environment and the contractile stimulus (i.e. number and strength of contractions). However, a recent study by Sylow et al. (2017) demonstrated that while muscle-specific knockout of Rac1 and AMPK impaired contraction-stimulated glucose uptake, this impairment was not observed with *in vivo* treadmill exercise (Sylow *et al.*, 2017). Hence, it will be interesting in future work to study the *in vivo* importance of SIRT1 to contraction-stimulated glucose

uptake, including during longer duration exercise. Furthermore, SIRT1 is one of eighteen known deacetylases in mammalian cells, so it will be of benefit in future work to study whether other specific deacetylases or classes of deacetylases are important to contraction-stimulated glucose uptake.

In conclusion, we investigated whether muscle-specific knockout of SIRT1 deacetylase activity reduces contraction-stimulated glucose uptake. Our results demonstrate SIRT1 deacetylase function is not required for contraction-mediated glucose uptake in adult mouse skeletal muscle. Interestingly, similar to findings related to insulin action, we did find sex differences in contraction-stimulated glucose uptake, such that it was ~30% higher in female versus male, regardless of muscle type. The goal of future work will be to identify the molecular mechanisms that underlie these sex difference in contraction- and insulin-stimulated glucose uptake.

MATERIALS AND METHODS

Animals

Mice with a muscle-specific knockout of SIRT1 (mKO) were generated using Cre-LoxP methodology, as previously described (Schenk *et al.*, 2011). Briefly, floxed mice harboring loxP sites flanking exon 4 of the SIRT1 gene were crossed with mice expressing Cre recombinase under the muscle creatine kinase promoter; deletion of exon 4, which encodes the deacetylase domain, results in a truncated SIRT1 protein that lacks deacetylase functionality (Cheng *et al.*, 2003; Philp *et al.*, 2011; Schenk *et al.*, 2011). Our breeding strategy was to breed a mKO (i.e. flox/flox, Cre-positive [one allele] littermates) with a WT (i.e. flox/flox, Cre-negative littermates). As such, litters produced both WT and mKO littermates and these mice were housed together with a limit of 5 mice per cage. Mice were housed on a 12:12-h light/dark cycle and had ad libitum access to chow (catalog no. 7912, irradiated; Envigo Teklad) and water. All studies were conducted in male and female mKO (Cre-positive) and WT (Cre-negative) littermates at ~12-weeks of age. Experiments were carried out with the approval of, and in accordance with, the Animal Care Program and Institutional Animal Care and Use Committee at the University of California, San Diego.

Muscle dissection and ex vivo electrical stimulation

After a 3 h fast, mice were anesthetized (ketamine, 25mg/kg; acepromazine, 1mg/kg; xylazine, 2 mg/kg) via intraperitoneal injection. Once anesthetized, suture (size: 6-0) were tied at the myo-tendinous junction at each end of the extensor digitorum longus (EDL) and soleus and the muscles from both legs were removed and pre-

incubated (20 minutes, 35°C) in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit buffer (KHB) containing 2 mM sodium pyruvate and 9 mM mannitol (PreInc-KHB). After the 20 min preincubation period, one soleus and one EDL were mounted in a specialized muscle chamber containing PreInc-KHB (25^{o}C), with continuous oxygenation (room air). The muscle origin was tied to a rigid post, and the insertion was secured to the arm of a dual-mode ergometer (model 300B; Aurora Scientific, ON, Canada). Muscles were stretched to optimal length based on resting tension (0.4V for soleus and 0.2V for EDL) in the pre-incubation buffer. The rationale for this criterion for setting optimal length was to prevent additional stimulation of the muscle, which in turn could impact glucose uptake. Specifically, to determine the resting tension at optimal length, in preliminary studies EDL and soleus were gradually lengthened and the corresponding tension (in volts) at which supramaximal stimulation produced maximal isometric tetanic force was calculated; soleus (n=7): 0.4±0.13, EDL (n=13): 0.2±0.08. After establishing resting tension, muscles were stimulated (100 Hz, 35V, 2s train, 0.2ms pulse) every 15s for 10 minutes (40 total contractions) via an electrical stimulator (model S88; Astro-Med, West Warwick, RI) and parallel platinum plate electrodes that extended the length of the muscle. Tension was recorded throughout the contraction protocol and specific force was calculated by normalizing muscle force to muscle physiological cross-sectional area (Mcn & Vernon, 1975; Narici *et al.*, 1992; Maganaris *et al.*, 2001). Fatigability was calculated as the time it took each muscle to reach a force that was 40% of the initial contraction.

***Ex vivo* 2-deoxy glucose uptake (2DOGU)**

Immediately after the last contraction, the contracted muscle and contralateral "rested" muscle were transferred to flasks containing KHB containing 1 mM 2DG, 8 mM mannitol, 2 mM Na-pyruvate, [^{14}C]-mannitol (0.053 mCi/mmol; American Radiolabeled Chemical (ARC)) and [3H]-2DG (3 mCi/mmol; ARC). After 10 minutes, the muscles were blotted on filter paper, trimmed, rapidly frozen in liquid nitrogen and stored (–80°C). 2DOGU rate was calculated as previously described (Schenk *et al.*, 2011; Martins *et al.*, 2019*a*, 2019*b*).

Muscle homogenization

Soleus and EDL were homogenized (Bullet Blender Tissue Homogenizer, Next Advance #BT24M) in 500µL ice-cold homogenization buffer (50 mm Tris, pH 7.5, 250 mm sucrose, 1 mm EDTA, 1 mm EGTA, 1% Triton X-100, 50 mm NaF, 1 mm $NaVO_2$ $Na_2(PO_4)_2$, and 0.1% DTT) containing phosphatase inhibitor cocktail (PIC) 2 (MilliporeSigma #P5726), PIC 3 (MilliporeSigma #P0044), Complete (MilliporeSigma #11836170001), 1 mM trichostatin A (Cell Signaling #9950S), and 1M nicotinamide (MilliporeSigma #N0636). After homogenization, muscles were rotated for 2 hours at 4°C and the supernatant was collected by after centrifugation (12,000 rpm/14,167 g) for 20 minutes at 4°C and then stored at -80°C for counting for 2DOGU and immunoblotting.

Immunoblotting

Lysate protein concentrations were quantified via bicinchoninic acid method (Pierce BCA Protein Assay Reagent A #23223 and Reagent B #23224) and then

samples were prepped to the same protein concentration (1μg/uL) in 1 × Laemmli sample buffer. After boiling for 5 min at 100°C, equal amounts of protein (20 μg) were separated on XT criterion precast gels (Bio-Rad Laboratories) under reducing conditions and were then transferred to nitrocellulose (Cytiva). The nitrocellulose membranes were stained with ponceau S solution (0.1% [wt/vol] ponceau S in 5% acetic acid) then washed with 1X TBST. Next, the membranes were blocked with 5% milk in TBST for 1 hour at room temperature and were then incubated overnight with gentle agitation in primary antibodies at 4°C. The following primary antibodies from Cell Signaling Technology were diluted 1:1000 in 5% BSA: p38 MAPK (CS 9212), phospho-p38MAPK (CS 9211), Phospho-AMPKα^{Thr172} (CS 2531S), Hexokinase II (CS 2867), Ac-Tubulin (CS 5335), and eEF2 (CS 2332). AMPKα1 and AMPKα2 primary antibodies were generously provided by Professor Grahame Hardie, University of Dundee. Following the overnight primary antibody incubation, membranes were incubated in appropriate secondary antibodies (1:10,000 in 5% milk in TBST) for 1 hour. The blots were developed utilizing a horseradish peroxidase chemiluminescent substrate (Bio-Rad Laboratories#1705061). Densitometric analysis of immunoblots was performed using Image Lab Software 6.1 (Bio-Rad Laboratories). Phosphorylated proteins were normalized to total abundance and total proteins were normalized to eukaryotic elongation factor 2 (eEF2) or Ponceau S.

Statistics

Statistical analyses were performed using Prism 8 (GraphPad Software Incorporated, La Jolla, CA, USA). All data were analyzed using an unpaired Student's

t-test or 2-way ANOVA followed by a Tukey's post-hoc test for pairwise comparison. For sex difference comparisons in c-stim 2DOGU, because there were genotype differences within each sex, data for mKO and WT were collapsed together. For assessing the sex difference in HKII abundance, data for mKO and WT as well as rested and contracted muscles were collapsed within each sex as there were no genotypic differences nor changes with contraction stimulation. Statistical significance for all the data was set at $p<0.05$ and are values are expressed as mean ±SEM.

This thesis, in whole, is currently being prepared for submission for publication of the material. Kang, Ji H.; Park, Ji E.; Dagoon, Jason.; Schenk, Simon. The thesis author was the primary investigator and author of this material.

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