EXAMINATION OF THE EFFECTS OF AMP-ACTIVATED PROTEIN KINASE ACTIVATION IN OBESE MICE

EXAMINATION OF THE EFFECTS OF AMP-ACTIVATED PROTEIN KINASE ACTIVATION IN OBESE MICE

By

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ABSTRACT

The obesity epidemic is an important global health concern. Obesity is associated with a number of diseases including type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), cardiovascular disease, and some cancers. Insulin resistance, a precursor to type 2 diabetes, is defined as an unresponsiveness of metabolic tissues to insulin, leading to long-term hyperglycemia and hyperinsulinemia. The fatty acid-induced model of insulin resistance indicates that an accumulation of lipid intermediates interferes with insulin signal transduction leading to insulin resistance. It is, therefore, important to examine means by which these lipid intermediates can be reduced to alleviate interferences in insulin signaling in the treatment of insulin resistance and type 2 diabetes. Exercise and metformin are two common interventions in patients with type 2 diabetes and obesity. They both commonly activate AMP-activated protein kinase (AMPK). AMPK contributes to a number of metabolic processes including increased glucose and fatty acid oxidation. However, the effects of AMPK activation on insulin sensitivity are currently not fully understood. This compilation of studies examined the insulin sensitizing effects of AMPK activation via metformin, exercise, and novel AMPK activator R419 in obese mice. In Chapter 2 we show that metformin increases AMPK phosphorylation of acetyl-CoA carboxylase (ACC) 1 Ser79 and ACC2 Ser212, resulting in increased fatty acid oxidation, decreased lipid content and improvements in hepatic insulin sensitivity. In Chapter 3 we show that exercise-induced improvements in insulin

sensitivity occur independent of AMPK phosphorylation of ACC phosphorylation sites and independent of lipid content in the liver. Finally, in Chapter 4 we show that R419 improves skeletal muscle insulin sensitivity independent of AMPK and lipid content but improves exercise capacity via a skeletal muscle AMPK-dependent pathway in obese mice. These findings suggest that future studies examining the effects of AMPK activation in obesity will aid in our understanding of the mechanisms of insulin resistance and introduce methods of prevention and treatment of obesity and type 2 diabetes.

THESIS PUBLICATIONS

1. Fullerton MD, Galic S, **Marcinko K**, Sikkema S, Pulinilkunnil T, Chen ZP, O'Neill HM, Ford RJ, Palanivel R, O'Brien M, Hardie DG, Macaulay SL, Schertzer JD, Dyck JR, van Denderen BJ, Kemp BE, Steinberg GR. (2013). Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. *Nature Medicine*. 19(12): 1649-54.

2. **Marcinko K**, Sikkema SR, Kemp BE, Fullerton MD, Steinberg GR. Exercise training improves liver insulin sensitivity independently of liver lipid content, inflammation and AMPK-Acetyl-CoA Carboxylase signaling. *Molecular Metabolism*. MOLMET-D-15-00108.

3. **Marcinko K**, Bujak AL, Lally JSV, Ford RJ, Wong TH, Smith BK, Kemp BE, Jenkins Y, Li W, Kinsella TM, Hitoshi Y, Steinberg GR. (2015). The AMPK activator R419 improves exercise capacity and skeletal muscle insulin sensitivity in obese mice. *Molecular Metabolism*. 4(9): 643-651.

OTHER PUBLICATIONS

1. Ford RJ, Fullerton MD, Pinkosky SL, Day EA, Scott JW, Oakhill JS, Bujak AL, Smith BK, Crane JD, Blümer RM, **Marcinko K**, Kemp BE, Gerstein HC, Steinberg GR. (2015). Metformin and salicylate synergistically activate liver AMPK, inhibit lipogenesis and improve insulin sensitivity. *Biochem J*. 468(1): 125-32.

2. **Marcinko K**, Steinberg GR. (2014). The role of AMPK in controlling metabolism and mitochondrial biogenesis during exercise. *Exp Physiol*. 99(12): 1581-5.

3. Bujak AL, Blümer RM, **Marcinko K**, Fullerton MD, Kemp BE, Steinberg GR. (2014). Reduced skeletal muscle AMPK and mitochondrial markers do not promote age-induced insulin resistance. *J Appl Physiol* (1985). 117(2): 171-9.

4. Samaan MC, **Marcinko K**, Sikkema S, Fullerton MD, Ziafazeli T, Khan MI, Steinberg GR. (2014). Endurance interval training in obese mice reduces muscle inflammation and macrophage content independently of weight loss. *Physiol Rep*. 2(5).

5. Galic S, Fullerton MD, Schertzer JD, Sikkema S, **Marcinko K**, Walkley CR, Izon D, Honeyman J, Chen ZP, van Denderen BJ, Kemp BE, Steinberg GR. (2011). Hematopoietic AMPK β1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. *J Clin Invest*. 121(12): 4903-15.

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I dedicate this thesis to my mom, dad and brother and leave you with my favourite quotation from Blessed Teresa of Calcutta:

"Life is an opportunity, benefit from it. Life is beauty, admire it.
Life is a dream, realize it. Life is a challenge, meet it.
Life is a duty, complete it. Life is a game, play it.
Life is a promise, fulfill it. Life is sorrow, overcome it.
Life is a song, sing it. Life is a struggle, accept it.
Life is a tragedy, confront it. Life is an adventure, dare it.
Life is luck, make it. Life is too precious, do not destroy it.
Life is life, fight for it." -Blessed Teresa of Calcutta

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ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
AccDKI	ACC1 Ser79 ACC2 Ser212 double knock-in
ACS	Acyl-CoA synthetase
ADP	Adenosine phosphate
AGPAT	Sn-1-acyl-glycerol-3-phosphate acyltransferase
AICAR	5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside
Akt/PKB	Protein kinase B
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AST	Aspartate aminotransferase
AMPK-MKO	AMPKβ1β2-muscle knockout
ATP	Adenosine triphosphate
β-HAD	β-hydroxyacyl CoA dehydrogenase
CaMKK	Calmodulin-dependent kinase kinase
cAMP	Cyclic AMP
CBM	Carbohydrate-binding module
ChREBP	Carbohydrate response element binding protein
CoA	CoenzymeA
CPT-1	Carnitine palmitoyl-CoA transferase-1
CREB	cAMP response element binding protein
CRTC2	CREB regulated transcription coactivator 2
Cs	Citrate synthase
DAG	Diacylglycerol
DGAT	sn-1,2-diacylglycerol acyltransferase
EDL	Extensor digitorum longus
Elovl	Fatty acid elongases
FA	Fatty acid
$FABP_{PM}$	Fatty acid binding protein located on the plasma membrane
FAS	Fatty acid synthase
FAT/CD36	Fatty acid translocase/cluster of differentiation 36
FATP1-6	Fatty acid transport proteins 1-6
FOXO1	Forkhead box protein O1
G6Pase	Glucose-6-phosphatase
GK	Glucokinase
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GPAT	sn-1-glycerol-3-phosphate acyltransferase
GS	Glycogen synthase
GSK3	Glycogen synthase kinase 3

HDAC5	Histone deacytelase 5
HK	Hexokinase
HMGR	HMG-CoA reductase
HNF-4α	Hepatic nuclear factor 4α
IKK-β	IkappaB kinase-β
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	c-jun terminal amino kinase
KD	Knockdown
KI	Knockin
KO	Knockout
LCAD	Long chain acyl-CoA dehydrogenase
LCFA	Long-chain fatty acid
LKB1	Liver kinase B1
LPA	Lysophosphatidic acid
MCAD	Medium chain acyl-CoA dehydrogenase
MCD	Malonyl-CoA decarboxylase
MKO	Muscle knockout
mTORC	Mammalian target of rapamycin-Rictor complex
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
nNOSμ	Neuronal nitric oxide μ
OCT1	Organic cation transporter 1
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphorylase
PAS	Phospho S/T Akt
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PEPCK	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinse
PGC-1α	Peroxisome proliferator-activated receptor γ co-activator-1α
PI3K	Phosphatidylinositol-3 kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PP2A	Protein phosphatase 2A
PPAR	Peroxisome-proliferator-activated receptor
PPAR-γ	peroxisome proliferator activated receptor-γ
R419	N-(1-(4-cyanobenzyl) piperidin-4-yl)-6-(4-(4-methoxybenzoyl) piperidine-1-carbonyl
RER	Respiratory exchange ratio
SCAD	Short chain acyl-CoA dehydrogenase

SCD	Stearoyl-CoA desaturase
SIK	Salt-inducible kinase
SOCS	Suppressor of cytokine signaling
SREBP-1c	Sterol-regulatory-element-binding protein-1c
TAG	Triacylglycerol
TBC1D1	TRE2-Bub2-CDC16 domain family member 1
TBC1D4	TRE2-Bub2-CDC16 domain family member 4
TAK1	Transforming growth factor-β-activated kinase-1
TCA	Tricarboxylic/Citric acid cycle
VLAD	Very-long chain acyl-CoA dehydrogenase
ZMP	5-aminoimidazole-4-carboxamide riboside monophosphate

CHAPTER ONE

INTRODUCTION

1.1 Epidemiology of obesity and type 2 diabetes

Worldwide obesity rates have doubled in the last 30 years. Currently, about 36.9% of men and 38.0% of women are overweight or obese [1], defined as a body mass index ≥25 kg/m^2. Obesity is associated with a number of diseases including type 2 diabetes and cardiovascular diseases, which are largely preventable and 3.4 million deaths each year are attributed to being overweight or obese [1]. The accumulation of fat in obesity leads to risk factors like abdominal obesity, glucose intolerance, and hypertension, which are commonly categorized under one title as Metabolic Syndrome [2,3]. Between 50 to 90% of patients with type 2 diabetes are overweight or obese [4] and obesity is the most important risk factor for the development of type 2 diabetes [5,6]. There are two main types of diabetes – type 1 and type 2, characterized by a deficiency in insulin production and improper use of insulin in the body, respectively. About 90% of diabetics have type 2 diabetes, the majority of who are obese adults. Prevalence rates of diabetes in adults are projected to increase from 6.4% in 2010 to 7.7% by 2030. Developed countries are expected to have a 20% increase in the number of adults with diabetes, while developing countries are expected to have a 69% increase between 2010 to 2030 [7]. By 2030, diabetes will be the 7th leading cause of death worldwide. Along with contributing to 1.5 million deaths worldwide, diabetes is associated with many other complications such as heart disease and stroke,

neuropathy, retinopathy, and kidney failure [8]. In addition, current overweight and obesity rates in children in developed countries lie at 23.8% in boys and 22.6% in girls and in developing countries at 12.9% in boys and 13.4% in girls [1]. Only recently have children started to develop type 2 diabetes, speaking to the need for greater research in the field.

1.1.1 Non-alcoholic fatty liver disease (NAFLD)

Obesity is the leading cause of non-alcoholic fatty liver disease (NAFLD), characterized by hepatic steatosis, or an accumulation of lipids or fatty acids in the liver [9,10]. There is a 3-14% prevalence of NAFLD in the general population [11], and patients with type 2 diabetes commonly present with NAFLD [12], indicating a close relationship between the two processes [13–17] that may occur as a result of excess accumulation of fatty acid-derived intermediates that impede normal insulin sensitivity [18,19] (discussed below). NAFLD refers to a spectrum of liver manifestations ranging from simple steatosis and non-alcoholic steatohepatitis (NASH), to more serious liver fibrosis and cirrhosis, and eventually liver failure [20]. The first in the spectrum of NAFLD is steatosis, characterized by infiltration of fatty acids in the liver (>55.6 mg/g triglyceride level in the liver). Steatosis is clinically benign and reversible [21]. About 20-30% of individuals with steatosis can develop steatohepatitis [22], which refers to fatty acid infiltration into the liver that is accompanied with inflammation and collagen deposition. NASH can range from mild, moderate, to severe. Steatosis and NASH are followed by four stages of fibrosis, with cirrhosis the final stage referring to

irreversible damage to the liver compromising its function [20,23] (for details about the grading of NAFLD see Appendix A).

1.2 Regulation of blood glucose by insulin

Insulin is a hormone secreted by the β cells of the pancreas that primarily regulates metabolic pathways of glucose uptake, gluconeogenesis (production of glucose from the liver), protein synthesis, and lipid synthesis. Postprandial secretion of insulin occurs in response to high glucose levels, which in turn inhibits catabolic pathways of glycogenolysis, protein breakdown, and lipolysis and activates anabolic pathways of glycogen synthesis, protein synthesis, and lipogenesis [24]. An important distinction should be made regarding the effects of insulin on skeletal muscle and adipose tissue versus the liver in order to maintain blood glucose levels. Insulin increases muscle and adipose tissue glucose uptake via insulin-stimulated glucose transporters and coordinately inhibits the production of glucose, otherwise known as gluconeogenesis, from the liver (Figure 1.1). A dysregulation in insulin signaling leads to the development of insulin resistance.

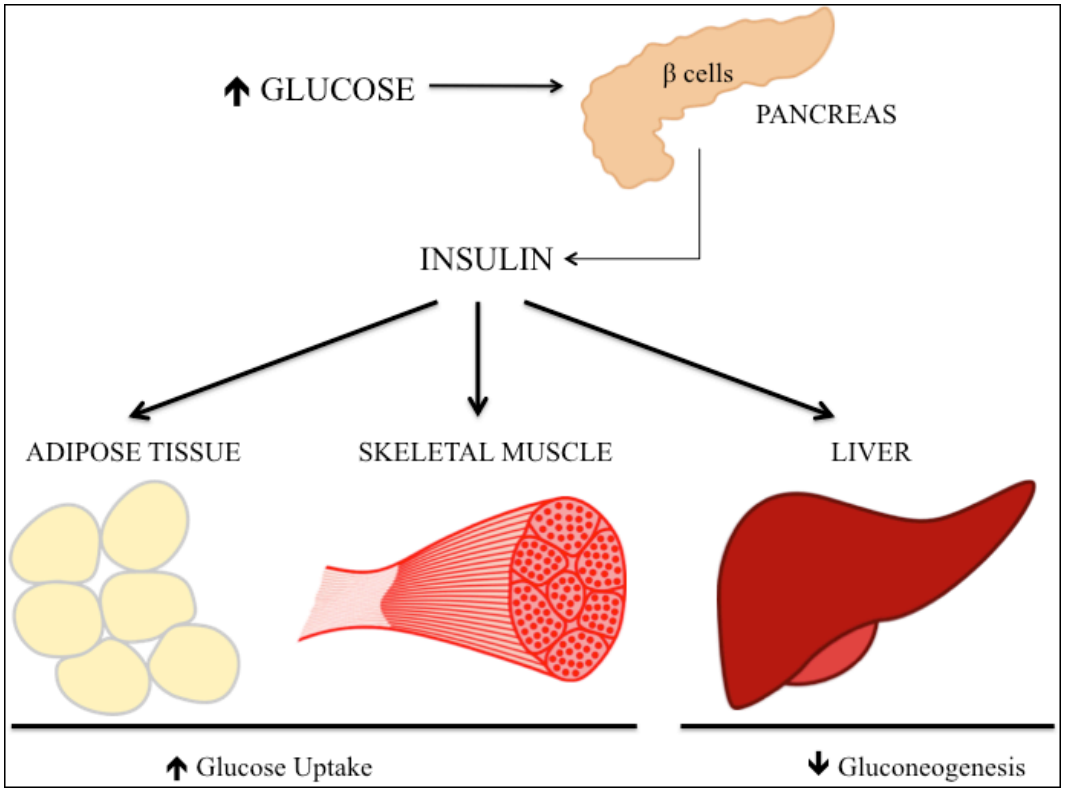

Figure 1.1 Regulation of blood glucose by insulin. In the fed state, increased blood glucose levels trigger insulin secretion by the pancreatic β cells, which increases muscle and adipose tissue glucose uptake and inhibits gluconeogenesis, (hepatic glucose production) from the liver.

1.2.1 Regulation of glucose uptake in skeletal muscle

In skeletal muscle and adipose tissue, the main function of insulin is to regulate glucose uptake, a process achieved by the translocation of glucose transporter 4 (GLUT4) to the plasma membrane. Insulin binds to its insulin receptor (IR) outside of the cell, leading to the activation of insulin receptor substrate (IRS)-1 and IRS-2 inside of the cell. The substrates then activate the catalytic subunit (p110) of phosphatidylinositol-3 kinase (PI3K) by phosphorylating its regulatory subunit (p85) [25,26] and results in the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to produce phosphatidylinositol-3,4,5-bisphosphate (PIP3). This leads to the recruitment of Akt/protein kinase B (PKB) and its conformational change, which causes Akt phosphorylation and activation at Thr308 via phosphoinositide-dependent protein kinase 1 (PDK1). Akt activation at Ser473 requires mammalian target of rapamycin-Rictor complex 2 (mTORC2) [27,28]. Akt phosphorylation of TRE2-Bub2-CDC16 domain family member 4 (TBC1D4) at Thr642 and phospho Ser/Thr Akt (PAS) sites leads to the translocation of GLUT4 transporters to the plasma membrane. GLUT4 release is believed to occur by TBC1D4 (previously known as AS160 (Akt substrate of 160 kDa)). Akt phosphorylation of TBC1D4 leads to inhibition of its GTPase activity and subsequent activation of Rab proteins due to GTP loading [24,29–32]. Akt inactivation of TBC1D4 results in GLUT4 translocation to the plasma membrane, which mediates glucose uptake. Akt also phosphorylates (Ser9) and inhibits glycogen synthase kinase 3 (GSK3),

which activates glycogen synthase (GS) and increases cellular levels of glycogen synthesis [33,34] (Figure 1.2).

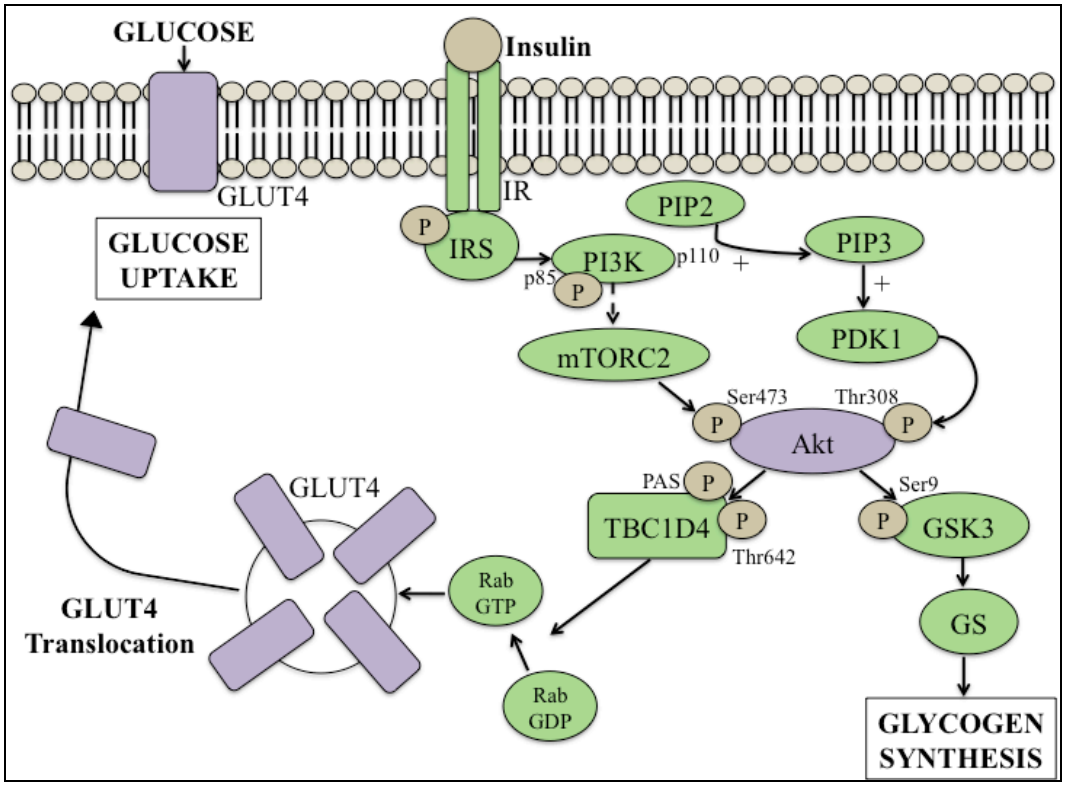

Figure 1.2 Regulation of glucose uptake by insulin. Insulin binding to the insulin receptor leads to elevated levels of glucose uptake and glycogen synthesis as described in detail above.

1.2.2 Regulation of liver gluconeogenesis

In the liver, insulin promotes glycogen synthesis and inhibits glycogenolysis (breakdown of glycogen) and gluconeogenesis (production of glucose from non-carbohydrate substrates like lipids or protein) [35,36]. When circulating insulin is low during fasting, liver glycogenolysis and gluconeogenesis are activated in order to maintain blood glucose levels. Fasting also increases circulating glucagon, which increases glycogenolysis. Glucagon binding to hepatocytes activates protein kinase A (PKA), which phosphorylates and activates glycogen phosphorylase kinase and glycogen phosphorylase, enzymes involved in glycogenolysis. Once glycogen stores are depleted in the liver, gluconeogenesis begins [35]. Transcription of gluconeogenic genes is regulated by the interaction of transcription factor cyclic AMP-inducible factor CREB (cAMP response element binding protein) and CREB regulated transcription coactivator 2 (CRTC2) [37]. Glucagon elevation during fasting leads to CRTC2 dephosphorylation and its translocation to the nucleus to upregulate CREB and other gluconeogenic enzymes [38,39]. CREB also activates gluconeogenesis by increasing peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) expression [37]. PGC-1α binds forkhead box protein O1 (FOXO1) and hepatic nuclear factor 4α (HNF-4α), which activates the transcription of gluconeogenic enzymes, like phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [40].

After feeding, blood glucose and insulin levels are elevated and glucagon low. Insulin binding to hepatocytes stimulates glycogen synthesis and turns off gluconeogenesis. Unlike GLUT4 in skeletal muscle and adipose tissue, GLUT2 is the predominant glucose transporter in the liver. GLUT2 is constitutively expressed on hepatocytes and circulating glucose enters hepatocytes through facilitated diffusion [36]. Once inside the cell, glucose is immediately phosphorylated by hepatic glucokinase (GK) and is subsequently stored in the form of glycogen. Insulin binding to the α subunit of its receptor leads to tyrosine phosphorylation of the intracellular β subunit and then tyrosine phosphorylation of IRS-1 and IRS-2, with IRS-2 as the main substrate in the liver [35,41]. IRS-2 activates PI3K by phosphorylating its p85 subunit. Pyruvate dehydrogenase kinase (PDK)-1 and Akt are then translocated to the plasma membrane and activated [35,42,43]. Akt increases glycogen synthesis by phosphorylating and inhibiting the activity of GSK-3 [35,42]. Akt phosphorylates FOXO1 at Ser253 [44], a transcription factor that inhibits binding to PGC-1α. This results in the down-regulation of gluconeogenic enzymes, like PEPCK and G6Pase [35,42]. Therefore, liver regulation of glucose homeostasis depends on a proper response to insulin in the fed and fasting state (Figure 1.3).

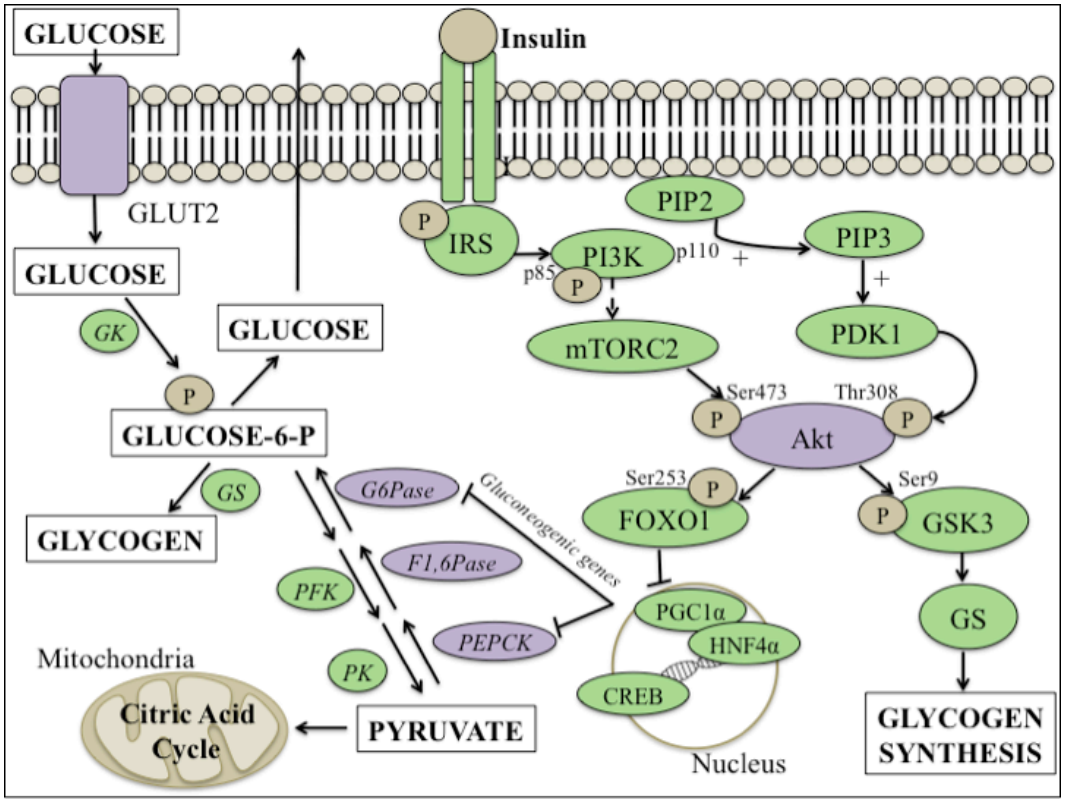

Figure 1.3 Regulation of gluconeogenesis by insulin. Insulin binding to the insulin receptor leads to inhibition of gluconeogenesis and increased glycogen synthesis in the liver as described above.

1.2.3 Insulin resistance

Insulin resistance, a precursor to type 2 diabetes [45], is defined as an unresponsiveness of metabolic tissues (skeletal muscle, adipose tissue, and the liver) to insulin. This unresponsiveness or resistance to insulin leads to long-term hyperglycemia and subsequent hyperinsulinemia in a compensatory response [24]. Despite extensive research, the exact mechanisms of insulin resistance are not

fully understood. Insulin resistance involves a dysregulation of the insulin signaling cascade downstream of the insulin receptor in metabolic tissues at many levels. Points of dysregulation include the insulin receptor [46,47] glucose transporter translocation [48–50], IRS-1 [51–53], IRS-2 [54,55], PI3K [56] and other insulin signaling substrates [57–60]. These points of modification, whether single or multi, ultimately result in impairments in skeletal muscle and adipose tissue glucose uptake and liver regulation of gluconeogenesis.

Skeletal muscle is especially important to the development of insulin resistance and type 2 diabetes because it regulates about 80% of insulin-stimulated glucose disposal [61,62]. In addition, the liver responds to fasting or feeding to regulate glucose levels using gluconeogenesis, glycogneolysis, and/or glycogen synthesis [35]. Lipid oversupply has been associated with impaired glucose disposal into skeletal muscle and adipose tissue [62] and impaired inhibition of liver gluconeogenesis [35] with insulin. This introduction will specifically examine mechanisms of fatty acid-induced insulin resistance in skeletal muscle and liver in relation to AMPK as a potential therapeutic target in the treatment of insulin resistance and type 2 diabetes.

1.2.4 Fatty acid/Lipid-induced insulin resistance

1.2.4.1 Randle cycle/Glucose-fatty acid cycle

In 1963, Randle and colleagues were the first to propose that insulin resistance occurred due to lipid/fatty acid-induced impairments in glycolytic enzymes within skeletal muscle, like pyruvate dehydrogenase (PDH) and phosphofructokinase-1 (PFK-1) [63]. PDH is involved in the conversion of pyruvate to acetyl-CoenzymeA (acetyl-CoA), which enters the citric acid cycle. Increased levels of fatty acids lead to increased levels of mitochondrial fatty acid oxidation. Fatty acids increase acetyl-CoA and NADH in the cytosol and increase citrate, which are inhibitors of PDH activity [64]. Furthermore, the metabolism of acetyl-CoA through the citric acid cycle leads to elevated levels of citrate, which inhibits PFK-1. PFK-1, the rate-limiting enzyme of glycolysis, converts fructose 6-phosphate and ATP to fructose 1,6-bisphosphate and ADP. PFK-1 inhibition leads to an accumulation of G6P due to feedback inhibition of hexokinase (HK) and glucose uptake [65]. Overall, this inhibition of glycolysis by fatty acids results in the synthesis of glycogen and conversion of pyruvate to lactate. Lactate is then converted back to glucose in the liver via the Cori cycle. Therefore, the glucose-fatty acid cycle proposed by Randle suggested that lipids inhibit glycolytic enzymes, leading to impairments in glucose metabolism (Figure 1.4).

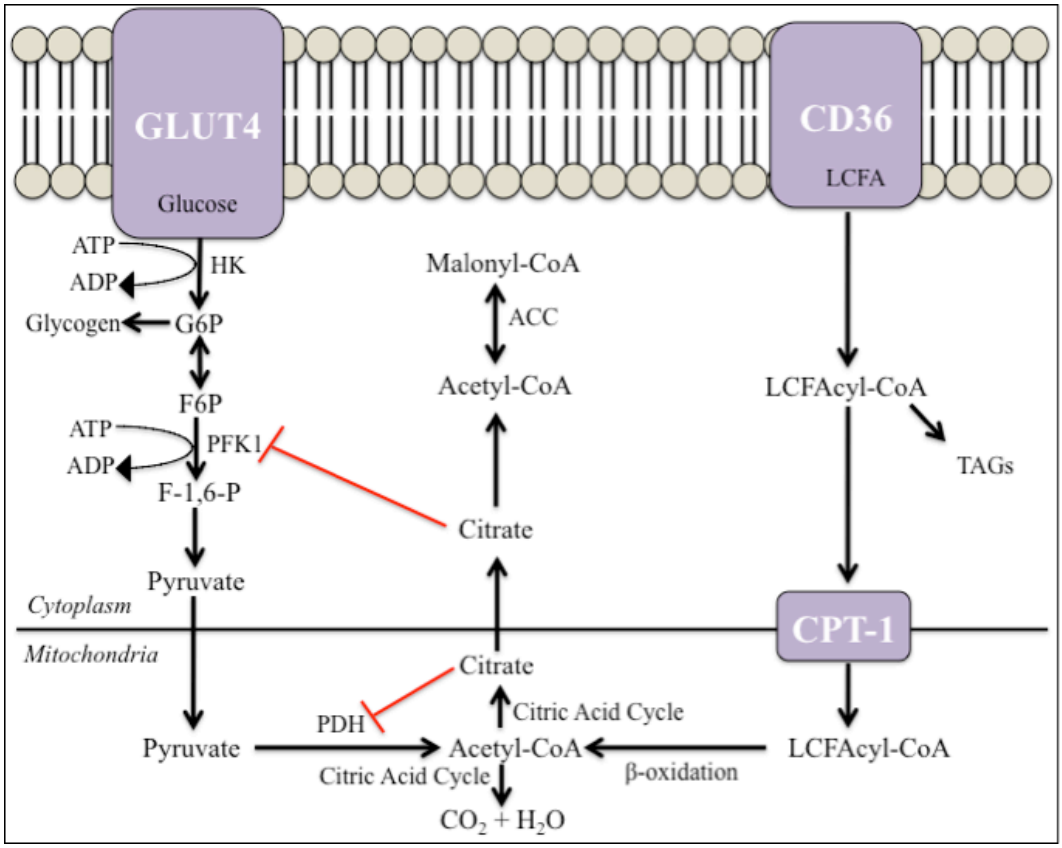

Figure 1.4 Glucose-fatty acid cycle. Randle and colleagues attributed insulin resistance to lipid/fatty acid-induced impairments in glycolytic enzymes within skeletal muscle, like pyruvate dehydrogenase (PDH) and phosphofructokinse-1 (PFK-1). The mitochondrial acetyl-CoA/CoA ratio increases and reduces acetyl-CoA production from pyruvate. The resulting citrate inhibits glycolytic enzymes, leading to a buildup of G6P and a reduction in glucose uptake by the cell.

1.2.4.2 Fatty acid-induced insulin resistance in skeletal muscle

The Randle cycle was later challenged with evidence to suggest that, rather than impeding glycolysis, high fatty acid concentrations cause impairments in glucose transport into skeletal muscle [66–68], an effect likely occurring due to reduced GLUT4 translocation to the plasma membrane [69,70]. It was later observed that fatty acids induced impairments in the phosphorylation and activity of IRS-1 or PI3K in the insulin signaling cascade [68,71,72]. Consequently, insulin resistance was proposed to develop in response to an accumulation of lipid intermediates like long-chain acyl-CoA (LCACoA), diacylglycerol (DAG), and ceramides in insulin-responsive tissues [17,73]. LCACoAs are activated free fatty acids within the cell [74] that have been associated with inhibiting hexokinase activity in skeletal muscle and activation of a family of protein kinase C (PKC) isoforms [75]. DAG and ceramides also activate PKC isoforms [76–79], along with IkappaB kinase-β (IKK-β) [80], c-jun terminal amino kinase (JNK) 1 [81], and suppressor of cytokine signaling (SOCS) [82], which impede the function of proteins downstream of the insulin receptor, like IRS and Akt (also referred to as protein kinase B). Increases in skeletal muscle PKC [83–85], IKK-β [86,87], JNK [88], and SOCS3 [82,89,90] have been associated with obesity and deletion of JNK1 [91] and SOCS3 [92] have been shown to prevent skeletal muscle insulin resistance.

DAGs are derived from triglycerides and phospholipids and act as a secondary messenger to activate PKC [93], while ceramides are derived from

phospholipids and they act as a secondary messenger to modify the activity of kinases, phosphatases, and transcription factors. The finding that high-fat diet increases skeletal muscle DAG and ceramide concentrations has been important in our understanding of insulin resistance [94]. PKCβ2, PKCδ [84] and PKCθ [84,95] induction by DAGs, in particular, have been implicated in skeletal muscle insulin resistance. For example, evidence has shown that PKCθ inhibits skeletal muscle IRS-1 activity [73], potentially through phosphorylation of IRS-1 at Ser307 [81]. Ceramides also accumulate in muscle in obesity and are believed to increase Akt dephosphorylation due to increases in protein phosphatase 2A (PP2A) [96] and increased Akt sequestration to caveolin-enriched microdomains, which prevents its phosphorylation [96,97]. Skeletal muscle Akt phosphorylation mediates the release of GLUT4 to the plasma membrane for glucose uptake (Figure 1.5A), so reductions in Akt phosphorylation will reduce GLUT4 translocation to the plasma membrane. Ultimately, reductions in IRS-1 and Akt activity within skeletal muscle may be an important mechanism by which DAGs and ceramides induce insulin resistance (Figure 1.5B).

Insulin resistance has also been associated with chronic low-grade inflammation [98–101]. As a result, examination of the SOCS family of proteins, which regulate inflammatory processes, has been important. SOCS proteins are elevated in a number of tissues in obesity. For example SOCS1 and SOCS3 expression are increased in the liver [102], adipose tissue [103–106] and muscle [82,90] of obese rodents. IL-6 [89,107], and tumor necrosis-α (TNF-α) [103] are

believed to trigger skeletal muscle SOCS3 expression. When elevated, SOCS3 has been shown to reduce insulin signaling by inhibiting the insulin receptor [106,108], IRS-1 or by reducing IRS tyrosine phosphorylation [102,109]. Together, these studies demonstrate several mechanisms by which lipid oversupply can contribute to the development of skeletal muscle insulin resistance; however, many other factors are also involved, thus highlighting the need for additional research in the area.

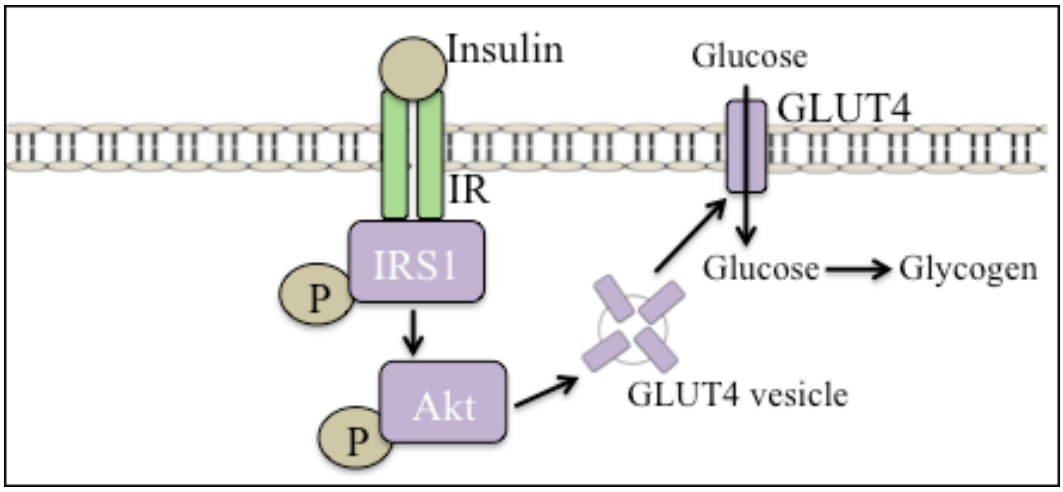

Figure 1.5 (A) Insulin-stimulated glucose uptake in skeletal muscle. Insulin-stimulated glucose transport into skeletal muscle is achieved via increased GLUT4 translocation to the plasma membrane.

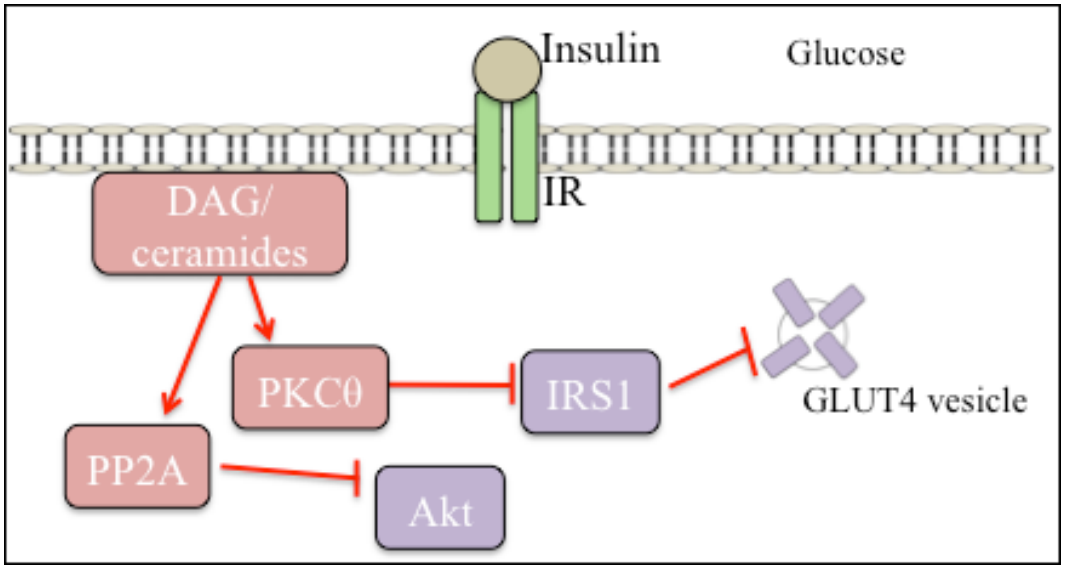

Figure 1.5 (B) Fatty acid-induced insulin resistance in skeletal muscle. Insulin resistance was proposed to develop in response to an accumulation of lipid intermediates like diacylglycerol (DAG), and ceramides in insulin-responsive tissues. DAG and ceramides activate a family of protein kinase C (PKC) isoforms. PKCθ has been shown to inhibit skeletal muscle IRS-1 activity and ceramides are believed to increase Akt dephosphorylation by protein phosphatase 2A (PP2A).

1.2.4.3 Fatty acid-induced insulin resistance in liver

The mechanisms by which elevated lipids contribute to liver insulin resistance have been extensively studied and likely involve the convergence of multiple pathways that inhibit insulin signal transduction [110,111]. There are currently a number of conflicting studies examining this relationship. Some studies regard lipid accumulation in hepatocytes as a cause of hepatic insulin resistance [112–114], while others, instead, suggest that type 2 diabetes leads to

fatty liver [115–123]. One of the goals of this thesis is to help shed some light on this discrepancy.

NAFLD and insulin resistance have both been associated with elevated visceral adipose tissue inflammation [124–129]. Visceral adipose tissue is believed to transfer free fatty acids and cytokines into the liver via the hepatic portal vein, referred to as the "portal effect", contributing to ectopic accumulation of fat in the liver [130].

Hepatic insulin resistance is associated with impaired inhibition of gluconeogenesis and increased glycogen synthesis in humans [131,132]. A number of rodent studies have examined the importance of lipid oversupply in the liver. Even a few short days of high-fat feeding leads to hepatic insulin resistance, characterized by a decrease in the phosphorylation of IRS-1 and IRS-2 by the insulin receptor [114]. A reduction in the phosphorylation of IRS will impair Akt function by reducing PI3K and PDK1 activity. Impairment in Akt will inhibit glycogen production via reductions in the phosphorylation of glycogen synthase kinase 3 (GSK-3) and glycogen synthase activity. Akt impairment will also increase FOXO1 translocation to the nucleus to upregulate gluconeogenic enzymes, thus, impairing insulin-stimulated inhibiton of gluconeogenesis [133] (Figure 1.6 A & B).

Similar to skeletal muscle, DAG activation of PKCs has been implicated in the development of hepatic insulin resistance. Compared to healthy individuals whose liver has no PKCε, a fatty liver is associated with high levels of PKCε

expression that impair insulin receptor kinase activity [134–139]. In addition, PKCε antisense oligonucleotide administration improves insulin signaling in rats fed a high-fat diet [139] and human data indicates that DAG expression of PKCε in the liver is strongly associated with liver insulin resistance [140] (Figure 1.4B).

While DAG and PKC activation have been strongly linked to the development of hepatic insulin resistance, it is unlikely that a single pathway directly contributes to insulin resistance (for review see [141] (Fu et al. 2012)). Indeed, many other proteins upregulated by fatty acids have been associated with impaired insulin action in the liver, including components of the endoplasmic reticulum-stress pathway, IKK-β, JNK1, PP2A [20,142] and SOCS molecules, which can also inhibit insulin signal transduction (for review see [143] (Galic et al. 2014)). Deletion of SOCS3 from the liver improves insulin sensitivity when fed a normal chow diet [144,145]; however, SOCS3 liver knockout (KO) mice that are aged or fed a high-fat diet, develop exacerbated insulin resistance and hepatic lipid accumulation [144,145], suggesting that SOCS3 is essential to prevent insulin resistance. Ultimately, the fatty acid-induced insulin resistance refers to impairments in insulin's ability to activate glycogen synthesis and inhibit hepatic gluconeogenesis in the liver, effects that have been observed in patients with type 2 diabetes [131,132] (Figure 1.6 B).

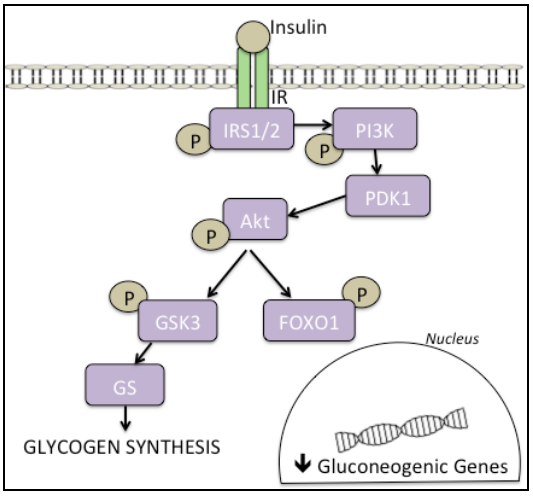

Figure 1.6 (A) Insulin-induced inhibition of gluconeogenesis. Insulin binding to its insulin receptor on the hepatocyte leads to inhibition of gluconeogenesis and favours glycogen synthesis.

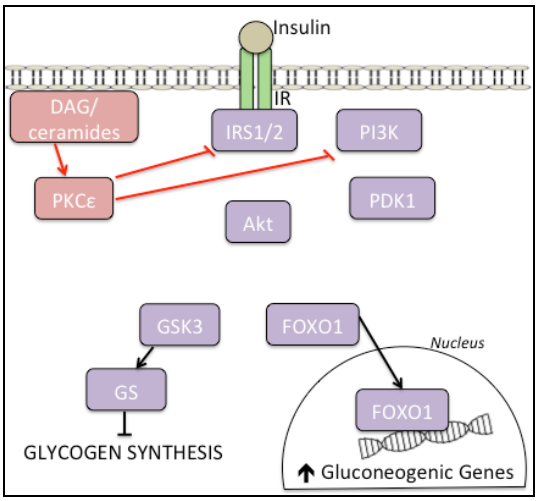

Figure 1.6 (B) Fatty acid-induced insulin resistance in liver. Hepatic insulin resistance was proposed to develop in response to an accumulation of lipid intermediates like diacylglycerol (DAG) activation of a family of protein kinase C (PKC) isoforms. PKCε has been shown to inhibit skeletal muscle IRS-1/IRS-2

and PI3K activity. The resulting suppression of Akt phosphorylation contributes to an inhibition of glycogen synthesis and activation of gluconeogenesis.

Taken together, the fatty acid-induced model of insulin resistance indicates that an accumulation of fatty acid-derived metabolites (DAG, fatty acyl-CoA and ceramides) correlates with decreases in insulin action and inactivation of the IRS-associated PI3K pathway. It is, therefore, important to examine means by which these lipid intermediates can be reduced to alleviate interferences in insulin signaling in the treatment of insulin resistance and type 2 diabetes.

1.3 AMP-activated protein kinase (AMPK)

Exercise is an important intervention in patients with type 2 diabetes. While reduced levels of daily physical activity increases the incidence of type 2 diabetes [146–150], even one bout of exercise has been shown to improve insulin-stimulated glucose uptake in sedentary adults [151] and to increase glucose uptake by >40% [152]. Individuals with metabolic syndrome, type 2 diabetes, and obesity present with an impaired capacity for exercise [153] and have been associated with low basal expression of the AMP-activated protein kinase (AMPK) in skeletal muscle [154,155] and adipose tissue [156–158]. AMPK is a metabolic enzyme whose regulation will be discussed in detail in the following sections of this thesis. Exercise/muscle contractions increase the activity of

AMPK [159,160], which has led to the suggestion that AMPK may be important for mediating the beneficial effects of exercise in type 2 diabetes. Importantly, the activation of AMPK with exercise is noted not only in the contracting skeletal muscle [160–165], but also adipose tissue [162–166], and liver [162–166]. And while it appears that AMPK is important for mediating the acute effects of exercise/contractions to stimulate glucose uptake in skeletal muscle [167], whether AMPK is important for mediating the insulin sensitizing effects of exercise in obesity is not currently understood [168] and as such will be the focus of Chapter 3 of this thesis.

In addition to exercise, metformin is the most commonly prescribed therapeutic for type 2 diabetes in the world. Metformin is derived from a French lilac called *Galega officinalis* [169]. It was first shown to clinically lower gluconeogenesis in patients with type 2 diabetes [170] and later showed evidence of lowering liver lipid levels [18]. However, the mechanisms by which this occurs and its relationship with type 2 diabetes are unclear. Metformin inhibits complex I of the mitochondrial respiratory chain leading to increases in cellular AMP and ADP [171,172] and activation of AMPK [173]. However, whether AMPK is required for the effects of metformin to improve insulin sensitivity is currently not fully understood and will be the focus of Chapter 2 of the thesis.

1.3.1 Basic structure and function

AMPK is ubiquitously expressed in all tissues and activation primarily occurs with phosphorylation of its Thr172 site on the α subunit (reviewed in [174]

(Oakhill et al. 2012)). Complete germline deletion of AMPK has been shown embryonic lethal and all subunits are necessary for proper function and activation of the enzyme [175]. AMPK was discovered in 1973 having the ability to inhibit acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HMGR) in the liver [176,177], a process that occurs in response to a change in "adenylate energy charge" [178]. Subsequent studies determined that AMPK is an enzyme that is activated in states of low energy status in order to turn on catabolic processes and inhibit anabolic processes in the cell [179,180].

AMPK exists as an αβγ heterotrimer structure with an α catalytic subunit and two regulatory subunits, β and γ [181,182] (Figure 1.5). There are multiple genes for each subunit (α1, α2, β1, β2, γ1, γ2, γ3), allowing for 12 potential heterotrimers of AMPK [183]. AMPK α2 is mainly expressed in muscle [184], while AMPK α1 is mainly expressed in brain and fat [185,186]. Liver expresses both α1 and α2 [187], and expression of these isoforms are almost completely dependent on the liver AMPK β1 subunit [188]. AMPK β2 is mainly expressed in muscle, with low levels in the liver and brain [189,190] and AMPK β1 is mainly expressed in the liver and brain, with low levels in the kidney and muscle [190]. As such, AMPK β1 KO mice have reduced AMPK activity in liver but not skeletal muscle [188] while AMPK β2 KO mice have reduced AMPK activity in skeletal muscle but not other tissues [191]. AMPK γ1 is expressed in all tissues

[186] while AMPK γ2 and γ3 are expressed primarily in the heart [192], and skeletal muscle [183,192–194], respectively.

The AMPK α subunits contain the catalytic phosphorylation site (Thr172) on their N-terminal while the C-terminal of the α subunit binds the β subunit [195,196]. The β subunit is often referred to as the scaffolding subunit since it contains an α and γ subunit binding sequence that is essential to maintain the structure of the AMPK heterotrimer [197–200]. The β subunit also contains a carbohydrate-binding module (CBM) [201], which has been associated with AMPK regulation via glycogen [202]. In addition to its role in regulating glycogen, the CBM has been shown to be critical for controlling enzyme activity in response to the direct AMPK activator A769662 [203,204]. Specifically, Ser108 within the CBM of the β1 subunit has been shown to be required for activation by the direct AMPK activators A769662 [205], salicylate [206], EX229 [207] and MT 63-78 [208].

Finally, the γ subunit is referred to as a regulatory subunit due to its nucleotide-binding properties [209,210]. The two Bateman domains on AMPK γ bind nucleotides AMP, ATP and ADP to oversee the allosteric control of AMPK [186,209,210]. AMP, ADP, and ATP bind to the AMPK γ subunit competitively [203,209,210]. AMP and ADP binding induces a conformational change to AMPK enabling its activation and preventing its dephosphorylation by phosphatases [203,209]. The AMPK γ subunit has 4 potential binding sites for AMP, but only 3 are able to bind nucleotides since they contain a conserved

aspartate residue that can bind the ribose sugar of AMP [182]. Two out of these three AMP-binding sites are exchangeable with ATP and one is non-exchangeable [182]. Some evidence suggests that ADP may play a similar role to AMP in mediating AMPK activation by LKB1 [209–211]; however, most recent evidence suggests that although ADP does protect from dephosphorylation, ADP concentrations need to be 10 fold higher to trigger a similar potency to which AMP can enable activation of AMPK [212]. As such, the γ subunit of AMPK is essential for activating AMPK in responses to energetic stress induced by hypoxia, exercise, AMP analogs (AICAR, C13) or compounds that inhibit mitochondrial respiration and increase cellular AMP levels (metformin, resveratrol, berberine, hydrogen peroxide) (as reviewed in Hardie 2015 [213]).

Recent publications have described the crystal structures of human α2β1γ1 [214] and α1β1γ1 [215] heterotrimer complexes that were fully activated by phosphorylation on Thr 172 and crystallized in the presence of AMPK activators AMP, A-769662 or 991. AMPK contains two main components – the catalytic component and the nucleotide-binding component. The catalytic component contains the kinase domain and glycogen-binding domain, between which lies the binding site for A-769662 and 991. The nucleotide-binding component contains the C-terminals of the alpha and beta subunit and the entire gamma subunit with the regulatory subunits for AMP, ADP, and ATP [214,215] (Figure 1.7).

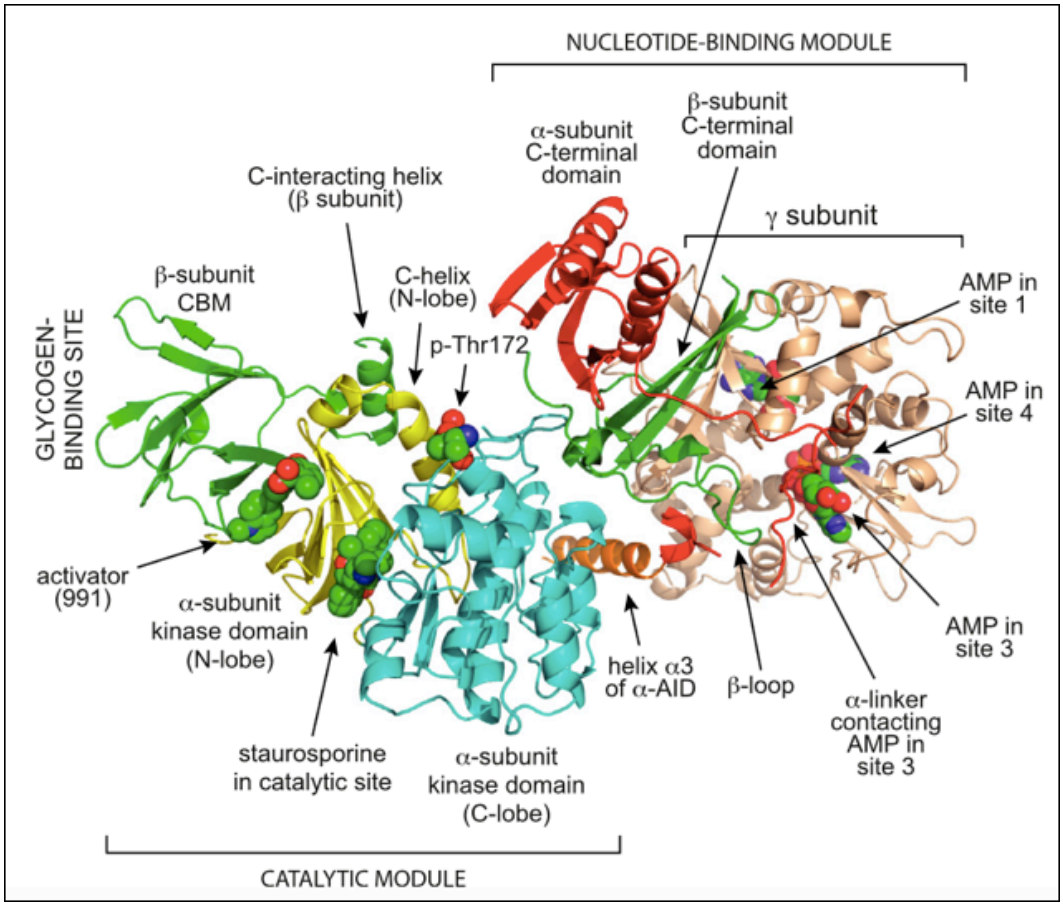

Figure 1.7 Structure of AMPK. AMPK exists as an αβγ heterotrimer structure with an α catalytic subunit (α1, α2) and two regulatory subunits, β (β1, β2) and γ (γ1, γ2, γ3). AMPK α2β1γ1 is 1 of 12 potential heterotrimers of AMPK illustrated in the figure. Structurally, the heterotrimer can also be divided into a catalytic module (bottom left) and a nucleotide-binding module (top right). The AMPK α subunits contain the catalytic phosphorylation site (Thr172) lying in the cleft between these two modules. The β subunit acts as the scaffolding subunit that maintains the structure of the heterotrimer by binding the α and γ subunit and the

γ subunit acts as regulatory subunit due to its nucleotide-binding properties. Figure taken from [216].

1.3.2 Regulation of AMPK activity by upstream kinases

Phosphorylation of AMPK Thr172 on the α subunit is the most potent activator of AMPK (>100 fold) (reviewed in [174] (Oakhill et al. 2012)). Myristoylation of the β subunit has been shown to be important for the phosphorylation of AMPK on Thr172; however, allosteric activation of AMPK by AMP (discussed in the following paragraph) does not require myristoylation [174]. There are three upstream kinases that have been shown to phosphorylate AMPK at Thr172: LKB1 [217–219], calmodulin-dependent kinase kinase (CaMKKβ) [203,220–222], and transforming growth factor-β-activated kinase-1 (Tak1) [223]. LKB1 is the major upstream kinase regulating AMPK function with the highest basal activity [218,224]. AMP binding to the AMPKγ subunit leads to conformational changes that favour LKB1 phosphorylation and activation of AMPK on its Thr172 site. In addition, LKB1 blocks AMPK dephosphorylation by protein phosphatase 2A and 2C (PP2A and PP2C) [203,209]. Recent studies have shown that AMPK activation by LKB1 during nutrient deficiency occurs at the surface of the lysosome, a process regulated by an adaptor protein axin, which brings AMPK and LKB1 to the Ragulator complex [225,226].

1.3.3 Exercise and metformin

Among its role in a number of other metabolic pathways, AMPK activation is a promising means to target and alleviate effects of fatty acid-induced insulin resistance in skeletal muscle and liver. Section 1.4 in this introduction will cover the role of AMPK in regulating skeletal muscle glucose uptake, exercise and mitochondrial capacity, fatty acid uptake and fatty acid oxidation. Section 1.5 will cover topics of liver gluconeogenesis and fat metabolism. Considering the ability for metformin and exercise to activate AMPK, the role of skeletal muscle and liver AMPK on insulin sensitivity and the influence of exercise and metformin will be covered in Section 1.6.

1.4 Skeletal muscle AMPK and exercise

Skeletal muscle is a highly dynamic tissue that can increase the rate of ATP turnover by >100-fold in response to exercise [227]. Under such conditions, AMP and ADP levels are increased in an intensity-dependent manner and ATP levels decline only slightly. Given the sensitivity of AMPK to changes in nucleotides, it is not surprising that AMPK is rapidly activated in response to muscle contractions (electrical stimulation) and exercise (cycling exercise in humans and treadmill running in mice) [159,165,228,229]. Interestingly, AMPK activation with exercise has a stronger correlation to changes in ADP, compared to AMP [174], as shown with direct binding studies which show free-bound ADP levels, but not AMP, reach levels required for AMPK Thr172 phosphorylation

[174,210,230]. Therefore, although exercise increases both AMP and ADP levels, ADP is responsible for the phosphorylation and activation of AMPK. The activation of skeletal muscle AMPK α1 and α2 is dependent on exercise intensity, with α2 AMPK activities increasing at moderate workloads starting at 40% of VO_2 Max and increasing progressively with higher intensity exercise. In contrast, AMPK α1 activity only appears to be increased during high intensity tetanic muscle contractions equivalent to >100% VO_2 Max [161,228,231]. In agreement with this idea, we have recently shown that skeletal muscle AMPK β subunits are critical for controlling exercise tolerance [167].

1.4.1 AMPK expression in skeletal muscle

AMPK plays a central role in skeletal muscle metabolism. Human skeletal muscle predominantly expresses the AMPK α2 and AMPK β2 subunits, 20% of which associate with AMPK γ3 and 80% of which associate with AMPK γ1 (α2/β2 and α1/β2) [232]. Out of all the potential AMPK heterotrimer complexes found in skeletal muscle in mice [233] and humans [234], exercise has been found to activate only the α2β2αγ3 and α2β2γ1 AMPK heterotrimer complexes [234–236]. Various isoform-specific mouse models have been examined to determine the importance of skeletal muscle AMPK. Whole-body AMPK α2 KO and AMPK α2 knockdown (KD) mice have reduced AMPK α2 activity and AMPK Thr172 phosphorylation in skeletal muscle [237–239]. AMPK α2 KO mice show enhanced activity of AMPK α1, while AMPK α2 KD mice show a slight

reduction in AMPK α1 in skeletal muscle. AMPK α1 KO mice have reduced AMPK α1 activity but normal AMPK α2 activity and AMPK Thr172 phosphorylation because AMPK α2 is the main isoform upholding AMPK activity in skeletal muscle [239]. Furthermore, AMPK β2 KO mice have reduced AMPK α1 and α2 expression despite higher levels of the AMPK β1 isoform [191], while AMPK β1 KO mice have normal levels of AMPK activity in skeletal muscle [188]. AMPK γ3 is expressed only in skeletal muscle [183,192,193]. AMPK γ3 is predominantly expressed in fast-twitch glycolytic muscle like the extensor digitorum longus (EDL) [240] and AMPK α1 and β1 are primarily expressed in slow-twitch oxidative muscle like the soleus [241]. In mice, AMPK β1 and β2 is predominantly expressed in EDL, while AMPK β1 is primarily expressed in the soleus associated with AMPK α2 [189]. AMPK γ3 and γ2 are mainly expressed in EDL muscle [183,194], while γ1, γ2, and γ3 are evenly expressed in gastrocnemius muscle, which comprises a mix of slow-twitch and fast-twitch fibers [183].

1.4.1.1 AMPK and glucose uptake in skeletal muscle

The role of AMPK in regulating skeletal muscle glucose uptake has been studied extensively over the last 2 decades [242–245]. Both AMPK α1α2-muscle knockout (-MKO) and AMPK β1β2-MKO mice have confirmed that AMPK is not required for insulin-stimulated glucose uptake [167,246]. However, skeletal muscle AMPK does increase GLUT4 translocation to the plasma membrane

independent of insulin, to stimulate glucose uptake in response to pharmacological activators such as 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) [247–250], EX229 [207] and caffeine [251]. This process has been proposed to be mediated by AMPK phosphorylation of TBC1D1, a GTPase activator protein (Rab-GAP) at Ser237 and Thr596 (Figure 1.8) [167,252–255]. It is interesting to note that although insulin stimulated glucose uptake is impaired in type 2 diabetes, AMPK-mediated glucose uptake is not [256], making it an important area of research.

The first studies examining the role of AMPK in regulating glucose uptake studied the compound AICAR which acts as a mimetic of AMP when it becomes phosphorylated to 5-aminoimidazole-4-carboxamide riboside monophosphate (ZMP) [247,257,258]. AICAR stimulation of glucose uptake in resting skeletal muscle was lost in α2 [238,259], β2 [191] and γ3 [194] null mice. AICAR-mediated increases in skeletal muscle glucose uptake have been shown to depend on the phosphorylation of TBC1D1 and TBC1D4, which in turn allows GLUT4 to travel to the plasma membrane [260,261]. While the effects of AICAR to robustly increase glucose uptake via an AMPK-dependent pathway are well established, surprisingly other AMPK activators (A769662, metformin, C13) appear to have negligible effects on increasing glucose uptake [233,262–264]. The reasons for these differences are not fully understood but may be related to activation of different AMPK heterotrimers or cellular localization.

GLUT4 expression in skeletal muscle has been shown to increase after an acute bout of exercise in patients with type 2 diabetes [265]. In addition, the acute effects of AMPK activation on GLUT4 expression have been examined using AICAR *in vivo*. A number of studies have shown that AICAR leads to increased GLUT4 levels and increased glucose uptake [266–269], an effect that is lost in mice lacking AMPK α2- or γ3-subunits [259,270]. However, since AICAR has many non-specific effects, future studies need to confirm whether AICAR may be mediating GLUT4 expression via other mechanisms [269].

Overexpression of GLUT4 in skeletal muscle has been associated with improvements in insulin action [271] and GLUT4 transgenic mice are protected from high-fat diet-induced glucose intolerance [272]. Similarly, mice with transgenic deletion of GLUT4 develop insulin resistance and glucose intolerance [273]. This speaks to the essential role of GLUT4 in the longterm regulation of blood glucose. Interestingly, exercise training has been associated with increased intracellular stores of skeletal muscle GLUT4 and increased expression of GLUT4 transcription factors [274]. In addition, chronic AMPK activation has been shown to increase the expression of GLUT4 [275–277], an effect that may be occur via phosphorylation of HDACs [275]. These findings highlight that AMPK activation has a role in GLUT4 expression in skeletal muscle.

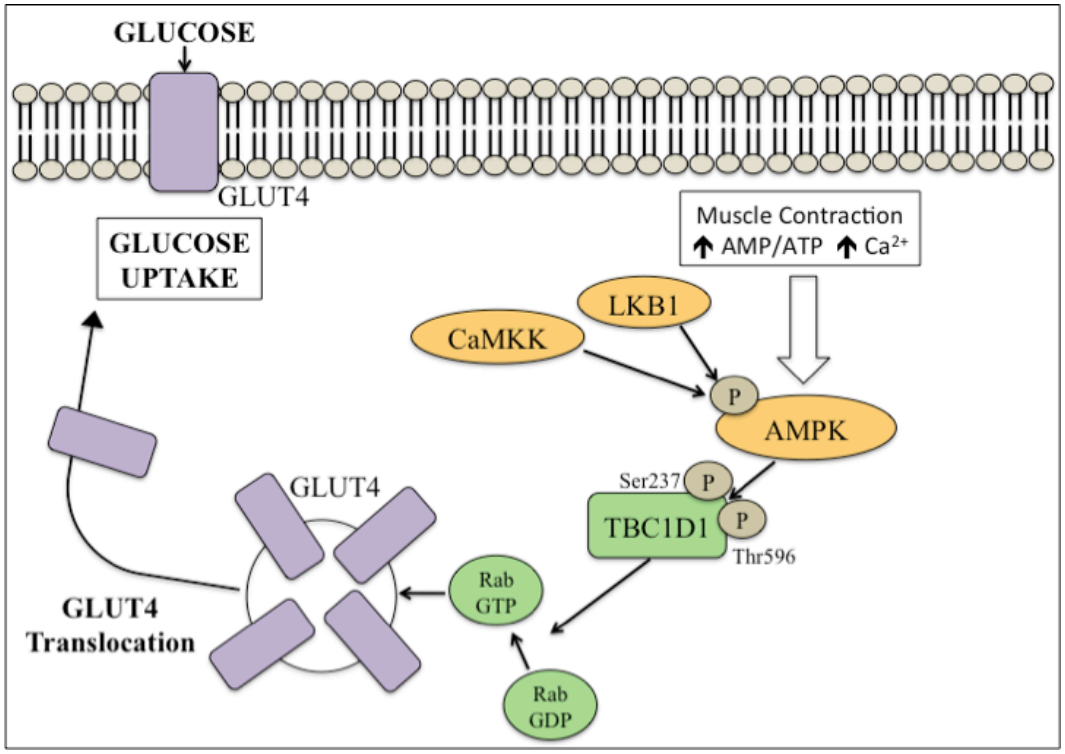

Figure 1.8 AMPK stimulation of skeletal muscle glucose uptake. AMPK regulation of skeletal muscle glucose uptake is proposed to occur via AMPK phosphorylation of TBC1D1, a GTPase activator protein (Rab-GAP) at Ser237 and Thr596.

1.4.1.2 Regulation of glucose uptake by AMPK during exercise/muscle contractions

The importance of AMPK for glucose uptake during muscle contractions has been examined extensively in genetically modified mouse models and has been shown to be necessary for contraction-stimulated glucose uptake in some [167] but not all studies [191,194,278,279]. It is now established that contraction-

stimulated glucose uptake can occur via a pathway that is independent of AMPKα2β2γ3 (the major heterotrimer found in muscle; [191,194,278,279]). However, a concern with studies in which only a single AMPK isoform is genetically altered/deleted is that there may be residual AMPK activity that is sufficient to increase glucose uptake during muscle contractions. Consistent with this idea, male mice lacking the AMPK β2 subunit specifically in skeletal muscle had normal glucose uptake (despite reductions in AMPK activity of >90%), but deletion of both AMPK β1 and β2 subunits simultaneously reduced muscle (soleus and EDL) glucose uptake during treadmill exercise and muscle contractions [167]. AMPKα1α2-MKO mice have a reduction in contraction-stimulated glucose uptake in the soleus but not extensor digitorium longus muscle [246]. The reason for the difference between studies is not currently known but may be related to the intensity of muscle contractions or the Cre-promoters used to drive deletion of AMPK subunits. Future studies investigating the mechanisms by which AMPK regulates glucose uptake during muscle contractions are warranted.

1.4.1.3 Skeletal muscle AMPK and its role in regulating mitochondrial biogenesis and exercise capacity

A number of studies have examined the importance of skeletal muscle AMPK in regulating exercise capacity. Numerous lines of transgenic mice with reductions in skeletal muscle AMPK have reduced exercise capacity/muscle

function [167,191,238,246,280–283]. As many of the studies have used a Cre-driven promoter that also reduces cardiac AMPK activity, it has been suggested that the poor exercise capacity with muscle AMPK deficiency may be due to reductions in AMPK activity in the heart which may impair cardiac function [284–287]. However, this is likely not the case as AMPKα1α2-MKO have reductions in exercise capacity despite normal cardiac AMPK activity thus indicating that skeletal muscle AMPK is indeed vital for maintaining exercise capacity [280]. Recent studies have suggested that one potential cause of reduced muscle performance may be a severe myopathy associated with reduced myofiber area, centrally located nuclei and increased split and necrotic myofibers [246,288]. In AMPKβ1β2-MKO, mice the myopathy was associated with increased platelet aggregation and a large reduction in capillary density and vascular perfusion, which was also noted in AMPK α2-KD mice, and may be mediated by AMPK regulation of neuronal nitric oxide μ (nNOSμ) [280]. Supporting this connection it has been found that AICAR treatment increased nNOSμ phosphorylation and nitric oxide production in WT but not AMPKβ1β2-MKO muscle cells, suggesting a role for skeletal muscle AMPK in regulating nNOSμ and potentially vascular perfusion. These findings suggest that AMPK is important for maintaining the vascular delivery of nutrients to skeletal muscle during exercise and that this may contribute to reduced exercise capacity/muscle performance. However, as muscles from AMPKβ1β2-MKO mice also fatigue more rapidly when contracted ex vivo, thus bypassing the need for vascular

delivery of nutrients, it is unlikely that reduced vascular perfusion is the only cause for the reduced muscle function.

Another mechanism by which AMPK may regulate exercise capacity involves its regulation of mitochondrial function. Exercise training has been known to increase mitochondrial biogenesis in skeletal muscle for many years [289–291]. One of the proposed mechanisms regulating mitochondrial biogenesis involves activation of the transcriptional co-activator PGC-1α, which increases the activity of the transcription factors nuclear respiratory factors 1 and 2 and mitochondrial transcription factor A [292]. Muscle-specific PGC-1α/β mice have a severe impairment in exercise tolerance and muscle oxidative capacity [293] that is similar to AMPK β1β2-MKO mice. Consistent with these findings, pharmacological or transgenic activation of AMPK has been shown to increase PGC-1α [234,294] and markers of mitochondrial function like citrate synthase (Cs) and β-hydroxyacyl CoA dehydrogenase (β-HAD) in many studies [295–297]. Pharmacological activation of skeletal muscle AMPK enhances mitochondrial biogenesis, an effect shown to be dependent largely on expression of the AMPK α2 subunit [283] and PGC-1α [298]. The overexpression of constitutively active skeletal muscle AMPK also increases PGC-1α and mitochondrial function, further supporting a critical role for this pathway in regulating mitochondrial biogenesis [299]. Many studies have investigated the mechanisms by which AMPK may regulate PGC1α activity, and this has been shown to involve both direct phosphorylation and acetylation (via sirtuin 1) of

PGC-1α (reviewed by O'Neill et al. 2013 [300]). Consistent with an important role for AMPK in regulating mitochondrial content, AMPKβ1β2-MKO and AMPKα1α2-MKO mice have reduced mitochondrial function [167,246]. Future studies investigating the effects of exercise on mitochondrial biogenesis in mice lacking both AMPK subunits in muscle will be important to establish whether factors other than the AMPK–PGC-1α–sirtuin 1 pathway are necessary for mediating adaptations to exercise.

1.4.2 AMPK regulation of fatty acid uptake in skeletal muscle

Fatty acid entry into skeletal muscle occurs through fatty acid transporters. These include the fatty acid binding protein located on the plasma membrane ($FABP_{PM}$) [301–303], fatty acid transport proteins 1-6 (FATP1-6) [230,304,305], and fatty acid translocase/cluster of differentiation 36 (FAT/CD36) [306]. FAT/CD36 is the primary contributor to fatty acid transport in skeletal muscle. Some data suggests that the transporter used for entry will determine the fate of the fatty acid. For example, FATP1 encourages fatty acid oxidation [307], while FAT/CD36 is believed to support fatty acid synthesis and storage in obese muscle [308]. Importantly, obese humans [309] and obese or insulin resistant rodents [310–314] have elevated rates of long-chain fatty acid (LCFA) uptake into skeletal muscle. In addition, increases in sarcolemmal FAT/CD36 compared to total FAT/CD36 protein expression have been observed in insulin resistant obese human myotubes [315] and a number of models of insulin resistance [311–

313,316]; however, the exact mechanisms are not known. Because obesity leads to elevated plasma free fatty acids, it is possible that increased localization of FAT/CD36 to the plasma membrane leads to greater uptake of LCFA [317], but further research is required.

Mice lacking AMPKα in skeletal muscle, have reduced protein expression of CD36 and $FABP_{PM}$ in soleus, quadriceps, and tibialis anterior muscles [318]. AMPK, fasting and muscle contractions have been shown lead to the recruitment of CD36 to the plasma membrane [319,320]. In addition to AMPK regulating CD36 localization/expression, it also appears that CD36 may regulate AMPK activity as a recent study has indicated that CD36 regulates LKB1 and AMPK activity by producing a CD36/Fyn/LKB1/AMPK protein complex [321]. When fatty acid availability is low, LKB1 relocates to the nucleus and as a result, AMPK remains inactive. Increased binding of fatty acids to CD36 leads to CD36 dissociation from Fyn, allowing LKB1 activation of AMPK [321]. Importantly, since CD36 variants have been associated with metabolic syndrome [322], poor regulation of this pathway may be a cause of metabolic complications in obesity.

Increased fatty acid uptake with muscle contractions is associated with relocation of the fatty acid transporter FAT/CD36 to the plasma membrane [323]. The AMPK activator AICAR increases FAT/CD36 translocation via an AMPK-dependent pathway; however, with muscle contraction, increases in fatty acid uptake occur via an AMPK-independent mechanism [323]. Recent studies have suggested that calcium/calmodulin-dependent protein kinases may be required

[324], an idea which is consistent with the very rapid translocation of CD36 to the plasma membrane at the onset of muscle contractions, before any detectable increases in AMPK activity [323].

1.4.3 AMPK regulation of fatty acid oxidation in skeletal muscle

Carnitine palmitoyl-CoA transferase-1 (CPT-1) is located on the outer membrane of the mitochondria and is a mediator of acyl-CoA transport into the mitochondria for oxidation [325]. High malonyl-CoA levels allosterically inhibit CPT-1. When malonyl-CoA levels are reduced (which occurs when ACC is phosphorylated and inactivated), the allosteric inhibition on CPT-1 is removed and this allows fatty-acyl-CoAs to enter the mitochondria for oxidation [326,327]. In addition to ACC, malonyl-CoA contents are also regulated by malonyl-CoA decarboxylase (MCD). MCD catalyzes the decarboxylation of malonyl-CoA thereby reducing cytoplasmic malonyl-CoA and enabling entry of fatty acids into the mitochondria for oxidation [328]. However, the importance of MCD remains questionable, and ACC may be the key enzyme that regulates the production of malonyl-CoA under most physiological conditions [329].

ACC has two different isoforms, ACC1 and ACC2. ACC1 is more highly expressed in liver and adipose tissue, while ACC2 is more common in heart and skeletal muscle [330]. Structurally, ACC1 and ACC2 are similar, but ACC2 has an additional NH_2-terminal allowing unique attachment to the mitochondrial membrane [331]. As a result, ACC2 was believed to be the primary regulator of

fatty acid oxidation, while ACC1 was believed to be the primary regulator of fatty acid synthesis [332]. However, this distinction has since become unclear and assessment of whether there is an overlap in function of the two enzymes is an important point of discussion in this thesis.

Given the predominant expression of ACC2 in skeletal muscle, the importance of this enzyme in regulating skeletal muscle and whole-body rates of fatty acid oxidation has been studied using genetic targeting. Initial findings in ACC2 KO mice showed that an ablation of ACC2 results in continuous fatty acid oxidation, less adipose tissue storage, reduced liver lipid and triacylglycerol content, and improved insulin sensitivity on normal chow [333,334] and high-fat and high-carbohydrate diets [138,334]. However, these findings were later challenged by two studies showing that ACC2 deletion has no effect on body weight, fat mass, and insulin sensitivity of mice [335,336]. The reason for the discrepancy between studies is not clear but it has been speculated that in the absence of ACC2 in skeletal muscle there may be upregulation of ACC1 and MCD that may compensate to control malonyl-CoA contents.

Transcription of ACC is regulated by a number of transcription factors including SREBP1c (sterol-regulatory-element- binding protein 1c), liver X receptor, retinoid X receptor, PPARs (peroxisome-proliferator-activated receptors), FOXO (forkhead box O) and PGC (PPARγ co-activator) isoforms [337–339]. Among the key transcription factors, SREBP1c plays a major role in controlling the expression of ACC1 and other lipogenic enzymes [339–341].

In addition to transcriptional control, ACC activity is also regulated through covalent and allosteric mechanisms. Insulin encourages the dephosphorylation and activation of ACC [342], an effect that occurs due to insulin activation of PP2A and inhibition of AMPK. This means that insulin favours an anabolic state in which fatty acid synthesis is active and fatty acid oxidation inactive. ACC activity is also increased allosterically by citrate. Citrate triggers polymerization of ACC, consequently, increasing its enzymatic activity [343]. Moreover, ACC enzyme activity is inhibited by covalent regulation of a number of phosphorylation sites [344]. AMPK and PKA have been shown to phosphorylate and inactivate ACC [333]. A number of studies have identified various phosphorylation sites that inhibit ACC and these include Ser79, Ser1200, Ser1215 (by AMPK), and Ser77 and Ser1200 (by PKA) [344–346]. Although the exact importance of PKA phosphorylation sites has never been established, Ser79 was determined to be the only AMPK phosphorylation site that was important for regulating ACC1 activity [347,348]. Ser79 exists on the ACC1 isoform, and is conserved on ACC2 as Ser221 [333]. AMPK, therefore, phosphorylates and inactivates ACC1 through phosphorylation at Ser79 [349–351] and it was assumed that paralagous phosphorylation of the Ser221 residue would inhibit ACC2 activity (Figure 1.9); however, this had not been genetically confirmed until our recent findings in ACC double-knock (KI)-in mice [352], which will be discussed in detail in Chapter 2.

MCD catalyzes the decarboxylation of malonyl-CoA, reducing cytoplasmic malonyl-CoA and enabling entry of fatty acids into the mitochondria for oxidation (Figure 1.9) [328]. AMPK has been shown to reduce cytoplasmic malonyl-CoA by phosphorylating and activating MCD [164,353]; however, whether AMPK is necessary for its regulation is not known [354]. ACC appears to be the primary enzyme controlling malonyl-CoA content under physiological conditions [329] but may involve a coordinate regulation of malonyl-CoA between MCD and ACC [164].

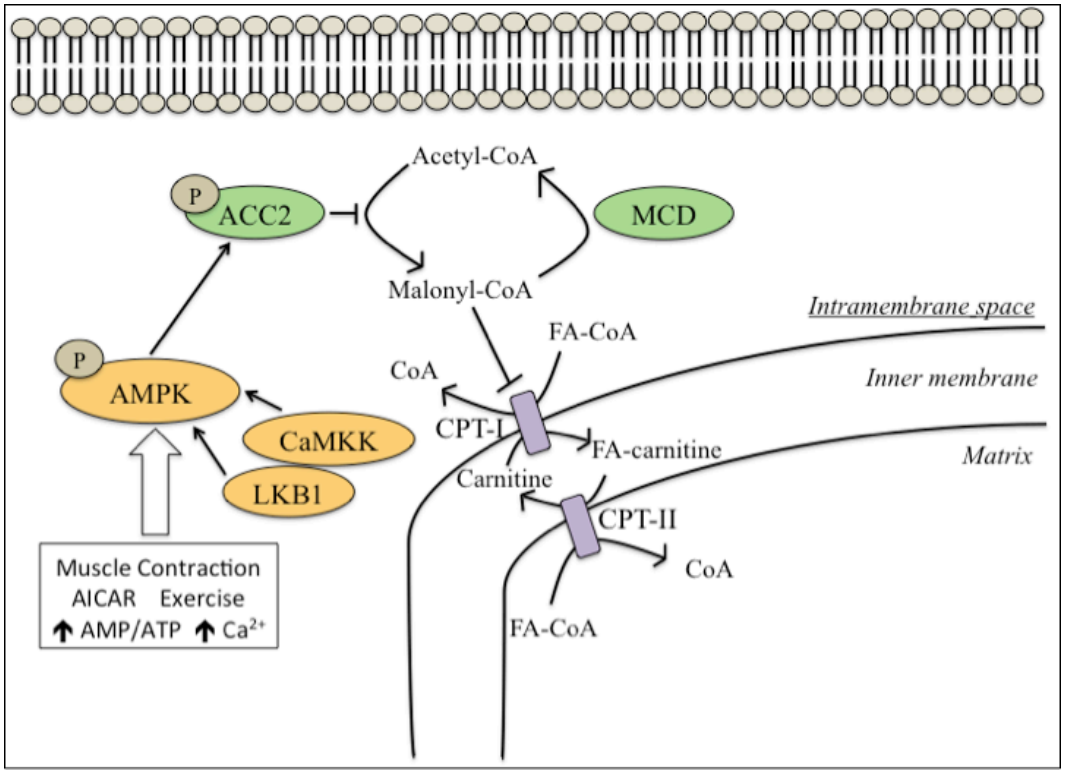

Figure 1.9 AMPK regulation of fatty acid oxidation. AMPK activation leads to the phosphorylation of ACC2 in skeletal muscle. This inhibits the conversion of acetyl-CoA to malonyl-CoA. MCD also catalyzes the decarboxylation of

malonyl-CoA to acetyl-CoA. The reduction in malonyl-CoA allows for fatty acid (FA) entry into the mitochondria to undergo β-oxidation.

1.4.3.1 AMPK and exercise in skeletal muscle fatty acid oxidation

Increases in fatty acid uptake into skeletal muscle with exercise and muscle contractions are accompanied by a reduction in the activity of ACC. The ACC2 isoform is phosphorylated at Ser221 in response to muscle contractions, an effect associated with reductions in ACC activity and malonyl-CoA and increased rates of skeletal muscle fatty acid oxidation [160].

The role of AMPK and ACC in regulating fatty acid oxidation during exercise/muscle contractions has been studied in both rodents and humans. In contrast to the observations detailed above, there appears to be a mismatch between AMPK activation, ACC2 phosphorylation, malonyl-CoA levels and rates of fatty acid oxidation. For example, it is well established that rates of fatty acid oxidation increase until ~65% of maximal oxygen uptake; however, AMPK and ACC phosphorylation are only somewhat increased at these moderate exercise intensities, and malonyl-CoA content is unchanged or only modestly reduced [355,356]. In contrast, during high-intensity exercise, where carbohydrates are used preferentially and absolute rates of fatty acid oxidation decline, AMPK is activated and ACC phosphorylation increased, while malonyl-CoA levels do not change [355]. These findings suggest that during muscle contractions and/or exercise, the concentration of malonyl-CoA is not vital for controlling mitochondrial fatty acid flux, a concept observed in a recent report detailing

insensitivity of mitochondrial fatty acid oxidation to malonyl-CoA in permeabilized muscle fibres [357].

Consistent with a potential AMPK/ACC2-independent pathway during muscle contractions/exercise, AMPK α2 kinase dead [237], AMPK β2 [191] and α2 null mice [358] display normal fatty acid oxidation during exercise/muscle contractions. A caveat of these studies is that these mice with partial AMPK deficiencies maintain the ability to phosphorylate and inhibit ACC2 during muscle contractions [191,237,358]. However a recent study in AMPKα1α2-MKO mice has shown a potentially important role for skeletal muscle AMPK in regulating fatty acid oxidation [246]. The authors find that AMPKα1α2-MKO mice preferentially utilize glucose instead of fatty acids as indicated by an elevated RER during treadmill running. Surprisingly, this finding is the opposite to what was observed in AMPK β1β2 MKO mice that had a lower RER compared to WT littermates when running at the same relative workload [167]. In addition, when skeletal muscle was examined *ex vivo*, fatty acid oxidation was impaired in AMPKα1α2-MKO muscle, suggesting an importance for skeletal muscle AMPK in regulating fatty acid oxidation during exercise.

As indicated, AMPK phosphorylates and inactivates ACC2 at Ser221. As a result, the role of AMPK in skeletal muscle fatty acid oxidation has been examined. Surprisingly, many mouse models with genetically induced reductions in skeletal muscle AMPK have normal rates of basal and AICAR-stimulated fatty acid oxidation, which may be due to the presence of residual ACC

phosphorylation [167,191,237,358]. The reason for this remaining ACC phosphorylation and preserved fatty acid oxidation in skeletal muscle but not in other tissues, such as liver [188] or macrophages [359], is not known and has raised the idea that skeletal muscle may contain an alternative ACC kinase [237]. Nevertheless, mice lacking skeletal muscle AMPKα show abnormal levels of genes involved in fatty acid oxidation, which may limit fatty acid oxidation *in vivo* [318]. Therefore, better examination of AMPK signaling of ACC on fatty acid metabolism in skeletal muscle is warranted.

In summary, this section discussed the role of skeletal muscle AMPK in regulating glucose uptake, exercise capacity, mitochondrial biogenesis, fatty acid uptake and fatty acid oxidation. AMPK is not essential to maintain insulin-stimulated glucose uptake. The importance of AMPK in controlling glucose uptake at different exercise/muscle contraction intensities and the mechanisms involved require further clarification based on conflicting reports in mouse models lacking skeletal muscle AMPK. The regulation of fatty acid uptake appears to be largely independent of AMPK. In resting conditions, AMPK regulation of ACC2 and PGC-1α in skeletal muscle has important roles in the regulation of fatty acid oxidation and mitochondrial biogenesis; however, future studies are needed to determine whether AMPK is required with exercise/muscle contractions. Given that disturbances in AMPK control of the above metabolic pathways are implicated in insulin resistance, a better understanding of these

processes during exercise may lead to improved therapeutic and preventative strategies for type 2 diabetes.

1.5 AMPK regulation of liver fatty acid metabolism

Similar to skeletal muscle, exercise has also been shown to activate AMPK in the liver [164,360]. Examination of AMP and ATP concentrations in liver and skeletal muscle following 35 minutes of endurance exercise and exhaustive exercise resulted in an interesting observation in which the liver appeared to be more susceptible to metabolic changes compared to muscle, as shown by a striking decrease in ATP concentrations and increase in AMP concentrations compared to muscle [361]. As a result of these findings, the topic of this section is to examine current knowledge on the role of AMPK activation in regulating liver metabolism and insulin sensitivity.

1.5.1 AMPK and liver gluconeogenesis

AMPK phosphorylates the carbohydrate response element binding protein (ChREBP), which inhibits the expression of the liver glycolytic enzyme pyruvate kinase [362]. AMPK also inhibits liver gluconeogenesis, an effect that has been shown to occur via phosphorylation of the coactivator CRTC2 at Ser171, and its subsequent exocytosis [363]. Similarly, salt-inducible kinase 1 (SIK1) and SIK2 are AMPK-related kinases that have also been shown to phosphorylate CRTC2 [363]. CRTC2 binding to 14-3-3 proteins when phosphorylated, prevents its translocation to the nucleus and CRTC2 regulation of gluconeogenic factors like

G6Pase and PEPCK [363]. Other means by which AMPK has been shown to inhibit gluconeogenesis include the phosphorylation of HNF-4α at Ser304 [364] and GSK-3β at Ser129 and Ser133 [365]. Phosphorylation of HNF-4α prevents DNA binding leading to its degradation [364]. In addition, AMPK phosphorylation and inhibition of GSK-3β leads to inhibition of gluconeogenesis via inhibition of the CRTC2 coactivator CREB [365] (Figure 1.10).

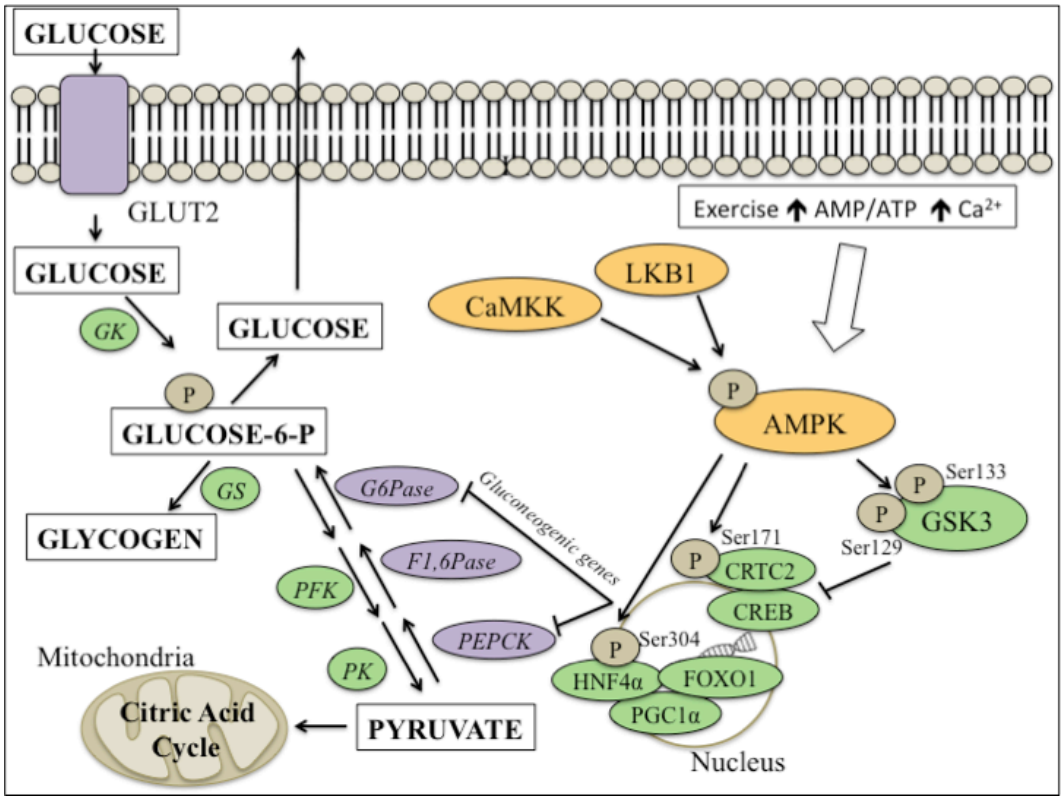

Figure 1.10 AMPK regulation of hepatic gluconeogenesis. AMPK inhibits liver gluconeogenesis, an effect that has been shown to occur via phosphorylation of the coactivator CRTC2 at Ser171 and phosphorylation of HNF-4α at Ser304. In addition, AMPK phosphorylation of GSK-3β at Ser129 and Ser133 leads to inhibition of gluconeogenesis via inhibition of the CRTC2 coactivator CREB.

1.5.2 Regulation of fatty acid metabolism in the liver

Liver sources for fatty acids include free fatty acids from the blood, chylomicron remnant uptake, and *de novo* lipogenesis (as reviewed by [366] (Mashek 2013)). Fatty acids are transported into the liver via FATP2, FATP5 [367,368] and the scavenger receptor CD36 [38], with a minor contribution from passive diffusion [369,370]. Importantly, CD36 is not required for normal rates of fatty acid uptake, since CD36 KO mice maintain normal rates of fatty acid uptake [38]; however, its overexpression has been associated with NAFLD in rodents and humans [38,371,372]. Once in the cytosol, fatty acid binding proteins (FABPs) attach to fatty acids for transport within the cell [373].

Insulin regulation of lipid metabolism involves increasing fatty acid synthesis and inhibiting fatty acid oxidation. Fatty acid synthesis is primarily controlled by sterol response element binding protein-1c (SREBP-1c), a transcription factor that regulates the expression of lipogenic (i.e. ACC, fatty acid synthase) and glycolytic (L-pyruvate kinase) enzymes in liver and muscle [374–376]. Insulin has been shown to activate SREBP-1c by initiating its cleavage in a PI3K-dependent manner leading to increased fatty acid synthesis [377]. Furthermore, insulin also regulates peroxisome proliferator activated receptor-γ (PPAR-γ). PPAR-γ is repressed by CREB during fasting to inhibit fatty acid synthesis, while insulin stimulation reduces CREB activity, thus, leading to PPAR-γ activation [37]. PPAR-γ is highly expressed in the liver, and when active, is involved in upregulating lipogenic genes [378,379]. The liver is believed to be

the primary tissue contributing to de novo lipogenesis in humans [380]. Importantly, liver *de novo* lipogenesis has been shown to be a critical cause of NAFLD in humans [381]; therefore, a better understanding of this pathway is important for developing new therapies for treating and preventing liver lipid accumulation. Rates of liver *de novo* lipogenesis vary dramatically depending nutritional status (fed versus fasted) [382]. During fed conditions, ACC and fatty acid synthase (FAS) are the key enzymes involved in *de novo* lipogenesis in the liver. As discussed in the previous section, ACC converts acetyl-CoA to malonyl-CoA, the first intermediate in the fatty acid synthetic pathway. FAS mediates the elongation of fatty acids to produce palmitate (16:0) [383]. Stearoyl-CoA desaturase 1 (SCD1) then generates C16:1 and C18:1 [384], long-chain FA elongase 5 generates C18:0 and C18:1 [385], and other elongases and desaturases can generate long polyunsaturated long-chain fatty acids (e.g. C18:2 and C18:3) [386,387]. Fatty acid activation is catalyzed by long chain acyl-CoA synthetase (ACSL) 3 and ACSL5. Acyl-CoAs can then be incorporated into triglycerides and/or phospholipids or can be oxidized by the mitochondria [388,389] (Figure 1.11 has a more in depth step description of fatty acid synthesis and oxidation).

In the first step of triglyceride synthesis, sn-1-glycerol-3-phosphate acyltransferase (GPAT) catalyses the conversion of glycerol-3-phosphate and acyl-CoA into lysophosphatidic acid. Sn-1-acyl-glycerol-3-phosphate acyltransferase (AGPAT) then catalyzes the conversion of lysophosphatidic acid into phosphatidic acid, which is then catalyzed by phosphatidylinositol to become

DAG. DAG catalyzation by sn-1,2-diacylglycerol acyltransferase (DGAT) creates a triacylglycerol [366].

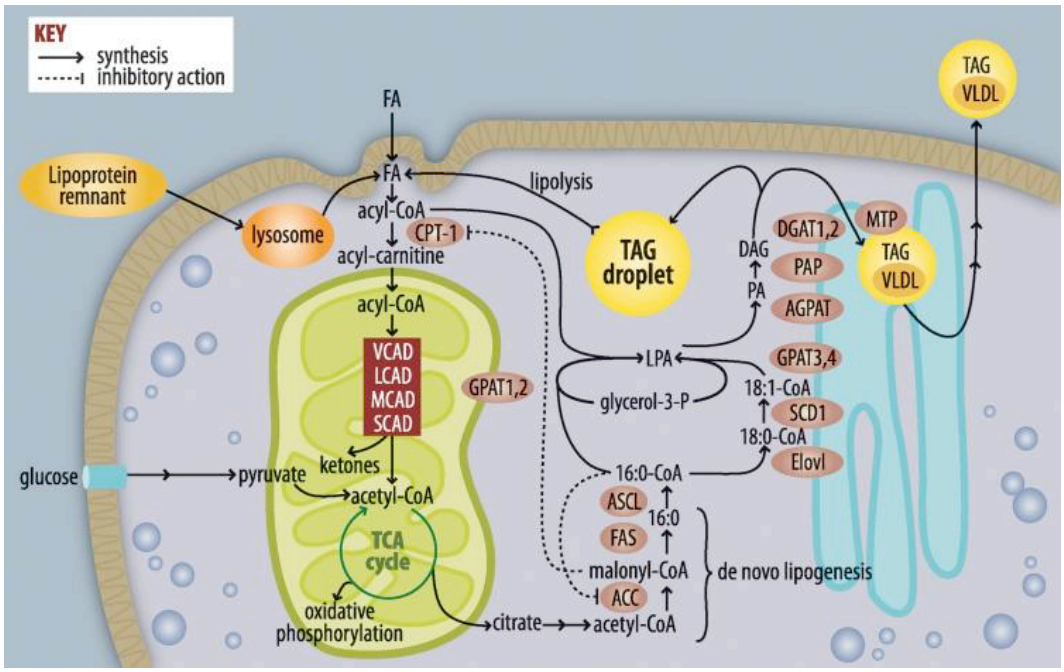

Figure 1.11. Triacyglycerol synthesis. Fatty acids are activated to form acyl-CoA, which can be used for either fatty acid synthesis or β-oxidation. In β-oxidation, acyl-CoAs are converted to acyl-carnitines that enter the mitochondria. Very-long chain acyl-CoA dehydrogenase (VLAD), long chain acyl-CoA dehydrogenase (LCAD), medium chain acyl-CoA dehydrogenase (MCAD), and short chain acyl-CoA dehydrogenase (SCAD) metabolize acyl-CoA into acetyl-CoA, which enters the tricarboxylic/citric acid cycle (TCA). The TCA cycle produces NADH and FADH, which are used for oxidative phosphorylation. In fatty acid synthesis, acetyl-CoA in the mitochondria is converted to citrate and citrate gets reconverted to acetyl-CoA once in the cytosol. *De novo* lipogenesis

begins when acetyl-CoA is converted to malonyl-CoA by ACC and fatty acid synthase builds malonyl-CoAs to make an acyl-CoA. Elongation of the fatty acids to form TAG requires fatty acid elongases (Elovl) and desaturases (SCD) and esterification of glycerol-3-phosphate by glycerol-3-P acyltransferases (GPAT) to create lysophosphatidic acid (LPA). LPA is then esterified to form phosphatidic acid (PA) by an acyl-glycerol-3-P acyltransferase. PA is hydrolyzed by phosphatidic acid phosphohydrolase (PAP) and finally, PAP is converted into TAG via diacylglycerol acyltransferase (DGAT). TAG is either transported into TAG droplets or VLDL for storage or transport, respectively (Figure from Nagle et al. 2009 [390]).

Under fasting conditions, when liver glycogen stores become depleted, free fatty acids and ketone bodies are used as the primary source of energy. Fasting may reduce fatty acid synthesis through liver AMPK phosphorylation and inhibition of the transcription of SREBP-1c, which in turn would lead to lower expression of lipogenic genes like FAS, mtGPAT, and ACC [164,391,392]. In addition to the transcriptional control of the lipogeneic program, AMPK also phosphorylates and inhibits ACC leading to reduction in malonyl-CoA. This in turn relieves allosteric inhibition of CPT-1 allowing long chain-fatty acyl-CoAs to be transported into the mitochondria for beta oxidation [393] while simultaneously reducing fatty acid synthesis (discussed in detail below) (Figure 1.12).

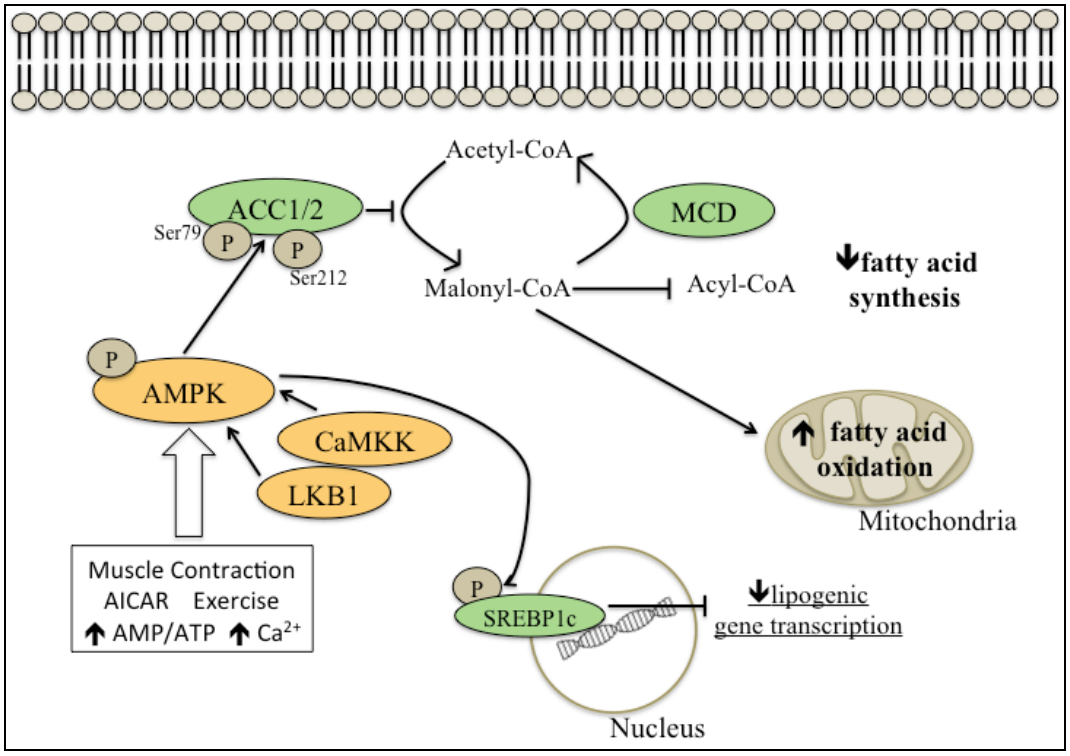

Figure 1.12 AMPK regulation of fat metabolism. AMPK decreases lipogenic gene transcription by phosphorylating SREBP-1c. Also, AMPK phosphorylation ACC1 at Ser 79 and ACC2 at Ser 212 leads to increases in fatty acid oxidation and decreases in fatty acid synthesis.

AMPK phosphorylation of ACC1 in the liver was the first substrate to be identified by Carling et al. in 1987 [394]. However, understanding the importance of AMPK in regulating this pathway has become more complicated as other points of regulation have been discovered. In particular, two isoforms of ACC (ACC1 and ACC2) have been identified, both of which are expressed in the liver. To date, the distinction between the roles of ACC1 and ACC2 and whether they maintain separate pools of malonyl-CoA has been unclear. The first study

published in this area showed reductions in fatty acid synthesis in liver the liver of the ACC1 KO mouse model, despite the maintenance of ACC2, thus suggesting that only ACC1 was capable of regulating fatty acid synthesis [395]. However, in another study in involving, this time using liver-specific ACC1 KO mice, liver fatty acid synthesis was normal, thus indicating ACC2 is able to compensate to maintain fatty acid synthesis in the liver [396]. Furthermore, inhibition of only ACC1 and ACC2 in rat hepatocytes by antisense oligonucleotides increased fatty acid oxidation in rat hepatocytes, while ACC1 suppression alone was sufficient to inhibit lipogenesis [397]. In the same study, inhibition of both ACC1 and ACC2 in rats was needed to significantly reduce hepatic malonyl-CoA and lipid content, and to improve hepatic insulin sensitivity [397]. These findings should be interpreted with caution since antisense oligonucleotides did not result in a complete reduction of ACC enzyme levels (~20% was still present). Given these differences between studies, it is still unclear about whether ACC1 and ACC2 have distinct (lipogenesis and fatty acid oxidation, respectively) or overlapping metabolic functions and is discussed in Chapter 2 of this thesis.

1.5.2.1 NAFLD and exercise in clinical studies

Recent clinical studies have shown that individualized exercise and diet interventions are beneficial at reducing liver lipid content or hepatic steatosis in adults [398–400] and adolescents [401–403]. In addition to reducing liver lipids, engaging in regular exercise is associated with a lower prevalence of NAFLD [404]. Findings of some studies, however, are not as supportive. Short-term

endurance training without weight loss does not change liver lipid content in men and women and 8 weeks of endurance training had no effect on alanine aminotransferase (ALT) and aspartate aminotransferase (AST), two markers of liver function [405]. Consequently, time and duration of exercise may play an important role in mediating changes in liver lipids. Vigorous [406], resistance [398], endurance [407], and aerobic exercise training programs [399,400] have all been associated with reductions in liver lipids. A careful analysis of how these types of exercises impact liver metabolism is necessary.

1.5.2.2 NAFLD and exercise in animal studies

Rodent studies have also recognized the importance of exercise in preventing the development of hepatic steatosis [408]. The duration and form of exercise is important to liver lipid accumulation because although an acute single 2 hour bout of aerobic exercise reduces intracardiomyocellular and intramyocellular lipids, it led to increases in intrahepatic lipid levels [409]. Nevertheless, studies examining training effects generally show reductions in hepatic lipid content [407,410,411] and cessation of training in previously active rats leads to the redevelopment of hepatic steatosis [412].

The effects of chronic exercise training on NAFLD have also been examined. Along with improving high-fat diet induced insulin resistance, 8 weeks of aerobic exercise (45 minute at a speed of 10 m/min) improved liver mitochondrial function and fatty acid oxidation, and reduced *de novo* lipogenesis and triglyceride synthesis [413]. Still, it is important to differentiate between types

of exercise and the related effects on lipid content in the liver. For example, Sene-Fiorese & colleagues compared the effects of 8 weeks of intermittent exercise (90 minutes/day, divided into three daily sessions (3 × 30 minutes/day) with 4 hour of controlled intervals between the exercise periods of swimming) and constant exercise (90 minutes/day swimming) to the development of hepatic steatosis. All groups were fed a high-fat diet and although both forms of exercise resulted in reduced liver lipid levels, intermittent exercise decreased the likelihood of developing fatty liver and NAFLD the most when compared to constant exercise. The rate of *in vivo* liver lipogenesis in the high-fat diet groups was also lowest in the intermittent exercise intervention [414]. Similarly, 8 weeks of high-intensity exercise (interval running sessions for 1 hour, consisting of successive 4 minute periods at 85–90% of VO_2 max, interspersed by 3 minute recovery periods at 70% of VO_2 max) in rats improved hepatic insulin resistance compared to continuous exercise (ran continuously at 70% of VO_2 max) [415]. Another study comparing liver lipid levels in rats that were genetically selected and bred for high aerobic capacity running versus low aerobic capacity running showed that high aerobic running capacity improved oxidative capacity in liver mitochondria [416]. In addition, the low-aerobic group of mice displayed higher lipid content in the liver, increased levels of SREBP-1c and ACC, and greater chance of lipid peroxidation – all characteristics of hepatic steatosis and oxidative stress [416]. A final study showed that vigorous-intensity interval treadmill running (twelve 1 minute intervals at 17 m/min with 2 minute active recovery at 10 m/min between

intervals), and moderate-intensity continuous treadmill running (8 m/min, giving a total exercise time of 46 minutes to match for distance) both improved NAFLD but it was vigorous-intensity interval treadmill running that was more efficient at improving hepatic steatosis [417]. Vigorous-intensity interval treadmill running was associated with greater AMPK activation and lower adiponectin and NF-κB secretion [417]. Therefore, evidence suggests an important role for type of exercise and that high-capacity intermittent exercise is likely more beneficial to hepatic liver lipid accumulation. Due to the variability in the exercise protocols used in studies, it is important to determine which form of exercise is most effective. Some studies, but not all, indicated that exercise was able to reduce liver fat content [398,418,419]; however, many of these studies showed exercise-induced weight loss [415,416,420–424]. Since weight loss is an important regulator of NAFLD, it is difficult to make a conclusion about the importance of exercise on regulating NAFLD.

1.5.2.3 Liver fatty acids, AMPK and exercise

Exercise is believed to regulate lipid content by increasing hepatic fatty acid oxidation and decreasing fatty acid synthesis [425], however, direct evidence supporting this hypothesis does not currently exist. Since AMPK regulates both of these metabolic processes, it is important to consider the role of AMPK activation in the liver to better assess the mechanisms of exercise-induced reduction of liver lipids. To shed some light on mechanism, one study suggested that glucagon action in the liver is essential to achieve reductions in liver lipid levels during

treadmill and running wheel exercise. High-fat feeding on mice with a deletion of the glucagon receptor did not show reductions in lipid levels in the liver as in wild-type littermates [426]. Interestingly, the glucagon receptor knockout mice did not show increases in AMPK phosphorylation; suggesting that glucagon-induced activation of AMPK was vital for reducing liver lipid content [426]. Two studies examining the impact of exercise cessation in previously trained animals observed a restoration of lipids in the liver [412,424]. Both studies also observed increases in ACC and FAS in livers of the exercise cessation group, suggesting greater lipid storage. This supports the hypothesis that exercise reduces fatty acid synthesis by AMPK phosphorylation and inhibition of ACC, which lowers cytosolic malonyl-CoA content to increase fatty acid oxidation. However, despite detection of ACC phosphorylation with exercise in these studies, AMPK phosphorylation was not detected in many studies [412,424]. This may be a result of tissue collection since AMPK phosphorylation is very sensitive to hypoxia and thus requires that liver tissue be snap frozen with liquid cooled tongs in situ [349].

In summary, this section summarizes the current information about the role of AMPK in regulating liver fatty acid metabolism and development of NAFLD. A number of clinical and animal studies have shown that exercise can reduce liver lipid content; however, the exact mechanisms remain unknown. Therefore a primary purpose of the thesis was to examine the importance of AMPK in regulating liver liver lipid metabolism and NAFLD.

1.6 AMPK and insulin sensitivity

AMPK has been shown to play an important role in regulating insulin sensitivity [427–429]. Glucose homeostasis requires both proper function of peripheral glucose uptake by skeletal muscle and adipose tissue and hepatic gluconeogenesis. The importance of AMPK has been studied in both skeletal muscle and the liver. This section will describe the findings of AMPK KO models and our current understanding about the role of AMPK in regulating insulin sensitivity in response to exercise training and metformin.

1.6.1 Liver AMPK and insulin sensitivity

Research has also suggested a vital role for liver AMPK in maintaining glucose homeostasis, effects which may be mediated in part through the control of liver gluconeogenesis. Cell studies initially showed an importance of AMPK in regulating gluconeogenesis [204,430]. The first *in vivo* studies indicating an important role for gluconeogenesis were made when mice lacking liver LKB1 (the upstream kinase of AMPK) developed severe hyperglycemia, an effect accompanied by a blunted activation of AMPK [431]. Similarly, genetic ablation of AMPK α2 from the liver leads to hyperglycemia and hyperinsulinemia [432]. These findings imply an importance of AMPK α2 in maintaining glucose production. Surprisingly, AMPK β1 KO mice are protected from high-fat diet-induced hepatic insulin resistance [188]. However, this was attributed to the hypothalamic effects which reduced food intake and weight gain in mice;

therefore, making it difficult to make any associations regarding the importance of AMPK in regulating hepatic insulin sensitivity.

The effects of exercise-induced activation of AMPK on hepatic insulin sensitivity have been examined in a limited number of studies. Mice fed a standard chow diet were given voluntary access to a running wheel for 8 weeks. Interestingly, improvements in hepatic insulin sensitivity occurred in the exercise group as shown by increased liver Akt Ser473 phosphorylation, increased insulin-stimulated GSK3β Ser9 phosphorylation, and reduced SOCS3 expression [421]. AMPK and ACC phosphorylation, which is associated with reductions in liver malonyl-CoA and lipid content, increased with exercise in mouse and rat liver in most [164,426,433,434], but not all studies assessing insulin sensitivity [360]. As a result, it is possible that AMPK may be mediating improvements in insulin sensitivity through phosphorylation and inhibition of ACC activity. Therefore, future studies examing the effects of exercise training in mice lacking the ability of AMPK to regulate ACC would help delineate the importance of liver AMPK in regulating insulin sensitivity following exercise training and is a topic that is covered in Chapter 3 of this thesis.

The liver contains high levels organic cation transporter 1 (OCT1) transporters making metformin particulary susceptible to uptake by hepatocytes [435]. A number of clinical studies have examined the effects of metformin on NAFLD [436]. Although metformin does not appear to alter liver histology clinically, it has been shown to improve insulin sensitivity and ALT and AST

levels in patients with hepatic steatosis [436]. In a clinical population, metformin was shown to only reduce glucose production without altering glucose uptake [437], and its main action was to lower hepatic gluconeogenesis in patients with type 2 diabetes [170]. AMPK was previously shown to be important for the action of metformin-induced improvements in insulin sensitivity [438] and hepatic steatosis [439,440]. Acute administration of metformin, however, was shown to decrease hepatic gluconeogenesis independent of liver AMPK in mice fed a normal chow diet [441], an effect occurring in response to reductions in glucagon induction of protein kinase A (PKA) [173]. In addition, inhibition of glucagon-mediated induction of gluconeogenesis in hepatocytes was shown to occur independent of AMPK [442]. Still, there is evidence to suggest that treatment of hepatocytes with metformin leads to reduced mRNA and protein expression of SREBP-1 and reduced ACC activity, an effect dependent on AMPK [173]. In order to better understand the mechanisms of metformin in the liver, an examination of the importance of fatty acid metabolism and how this relates to hepatic insulin sensitivity is necessary. Chapter 2 and Chapter 3 of this thesis will examine the importance of AMPK phosphorylation of ACC in mice fed a high-fat diet and treated with metformin.

As a result of the association between insulin resistance and NAFLD, it is important to look downstream of AMPK and examine the importance of AMPK regulation of ACC and fatty acid metabolism in association with hepatic insulin resistance. The impact of AMPK phosphorylation/inhibition of ACC on the

regulation of lipid metabolism and development on hepatic insulin resistance is a topic of Chapter 2 and Chapter 3 of this thesis.

1.6.2 Skeletal muscle AMPK and insulin sensitivity

Skeletal muscle controls ~80% of insulin-stimulated glucose disposal, indicating an important role for this tissue in maintaining glucose homeostasis [61,62,443]. AMPK activation by exercise has been shown to improve insulin sensitivity and glucose control in skeletal muscle [444] and reductions in skeletal muscle AMPK activity have been detected with aging and obesity in rodents [238,278] and humans [194]. These findings suggest an important role for skeletal muscle AMPK in sustaining insulin sensitivity. However, a number of skeletal muscle AMPK knockout mouse models do not support a vital role for AMPK in maintaining skeletal muscle insulin sensitivity when fed a control chow diet [167,239,246,445,446], an obesity-promoting high-fat diet [446,447], or when aged [445]. It should be noted that some studies have detected modest reductions in muscle insulin sensitivity [191,239,448]; an effect which may be due to increased catecholamines and not due to a direct defect in muscle metabolism [239].

A high-fat diet has been shown to impair the expression and activity of skeletal muscle AMPK in some [449] but not all studies [90,450]. In one study, the transgenic overexpression of a dominant negative AMPK $\alpha 2$ mice in muscle developed a more severe inhibition of insulin-stimulated glucose transport

compared to WT mice fed high-fat diet [451]. Similarly, AMPK β2 KO mice showed greater impairments in insulin sensitivity when fed high-fat diet compared to WT mice [191]. However, whole-body AMPK α2 deletion [446] and AMPK α2 kinase dead [447] mice fed a high fat diet did not show any difference in the development of high-fat diet-induced insulin resistance. Importantly, although high-fat diet decreased AMPK α2 mRNA in skeletal muscle, mRNA levels of AMPK α1 were not altered [449]. While previous studies have investigated the effects of an obesity-inducing high-fat diet on a genetic background of lower muscle AMPK activity, it is possible that small amounts of residual AMPK activity may have been sufficient to maintain skeletal muscle insulin sensitivity in these previous reports [446,447]. Data in Chapter 4 of this thesis sheds light on whether small amounts of AMPK may have been important in maintaining insulin sensitivity in mice fed a high-fat diet by examining insulin sensitivity in obese AMPKβ1β2-MKO mice.

Despite the equivocal results supporting a role for skeletal muscle AMPK activation to maintain insulin sensitivity, it should be noted that treatment of mice with many different types of AMPK activators consistently improve skeletal muscle insulin sensitivity [32,229,452–454]. However, the exact mechanisms by which AMPK may regulates skeletal muscle insulin sensitivity is currently not fully understood. Chronic exercise has been shown to improve skeletal muscle insulin resistance in obesity [455,456] but the exact mechanisms by which this occurs are not known. An increase in muscle insulin sensitivity has been shown to

persist many hours after an acute bout of exercise [457–461] and improvements in insulin sensitivity with chronic exercise have been attributed to increased AMPK in humans [462–464]. However, not all studies have shown a connection between activation of AMPK with exercise and improvements in skeletal muscle insulin sensitivity [459,465], suggesting other mechanisms are also likely involved.

Exercise-induced improvements in glucose control in skeletal muscle have been attributed to AMPK activation [444]. Although exercise training was shown to result in skeletal muscle metabolic changes independent of AMPK α2 on normal chow diet [283], 6 weeks of voluntary running wheel exercise did not improve skeletal muscle insulin sensitivity in AMPK α2 dominant negative mice as in WT mice fed a high-fat diet [466]. Briefly, AMPK α2 dominant negative mice given voluntary access to a running wheel showed partial improvements in skeletal muscle insulin sensitivity but did not show improvements in insulin levels, insulin-mediated palmitate uptake and oxidation, and CPT-1 expression that were achieved in WT mice. Still, reductions in palmitate oxidation, JNK1/2 phosphorylation, and CD36 expression occurred in both WT dominant negative mice [466]. This indicates that skeletal muscle AMPK α2 is at least partially required for insulin sensitization by exercise. However, it should be noted that although mice had equal running time, WT mice showed increased total running distance [466]. Future studies should control for distance traversed or work input during exercise between mice.

In addition to exercise training, the treatment of mice with a diverse range of compounds that activate AMPK including AICAR [248,467–469], resveratrol [470], berberine [452], metformin [263,453,471,472] or ciliary neurotrophic factor [90] has been shown to improve skeletal muscle insulin sensitivity. The insulin sensitization of these compounds have been shown to be mediated through skeletal muscle AMPK dependent [90,248,467,472] and independent [263,468,469] pathways.

Of the above listed compounds, metformin has been most intensely studied as it has been shown to increase skeletal muscle AMPK in insulin resistance rodents [449] and humans [473]. While there are some studies in muscle cells showing metformin may increase glucose uptake [263,471], clinically it has been shown that improvements in muscle glucose uptake or insulin sensitivity are usually not seen with metformin treatment in humans [474]. Instead the clinical data indicate the primary mechanism target for metformin action is the liver and suggest that studies showing effects in muscle may be primarily the result of the delivery method used (intraperitoneal administration vs. oral) and high concentrations studied which typically would not be observed in systemic circulation.

Based on the potential for muscle AMPK activators to improve exercise capacity and insulin sensitivity/glucose homeostasis, there has been a tremendous impetus from both industry and academic laboratories to develop new small molecule activators of skeletal muscle AMPK. Two direct activators of AMPK,

A769662 and salicylate, target the β1 subunit thus limiting their clinical utility in activating skeletal muscle AMPK [205,212] given the low expression of this subunit in skeletal muscle [188,191]; although it should be noted that recently EX229 has been shown to directly activate both AMPK β1 and β2 containing complexes in muscle [207]. Despite this, examination of chronic skeletal muscle AMPK activation in obesity is necessary to better elucidate effects of long-term AMPK activation on skeletal muscle insulin sensitivity. In Chapter 4 of this thesis we will discuss our findings related to R419 (N-(1-(4-cyanobenzyl)piperidin-4-yl)-6-(4-(4-methoxybenzoyl)piperidine-1-carbonyl)nicotinamide, which is a novel mitochondrial complex I inhibitor that activates AMPK in myotubes [475].

1.7 Main objective

The overall purpose of this thesis is to investigate the role of AMPK activation in obese mice.

1.7.1 Specific Aims

The specific aims of this thesis are to examine the effects of AMPK activation during the progression of diet-induced obesity in mice via:

1. Metformin (Chapter 2).
2. Exercise (Chapter 3)
3. Novel AMPK activator R419 (Chapter 4).

1.8 Hypotheses

In Chapter 2, we explore the effects of metformin on insulin sensitivity and fatty acid metabolism in AccDKI (serine-alanine knockin mutations of ACC1 Ser79 and ACC2 Ser212) mice generated on a C57Bl/6 background and WT mice fed a high-fat diet. AMPK phosphorylation of ACC activates fatty acid oxidation and inhibits fatty acid synthesis. Therefore, we hypothesize that AMPK activation by metformin treatment will lead to reductions in liver lipid levels in WT mice but not AccDKI mice. This will primarily occur in the liver due to the high levels of OCT1 transporter. This reduction in liver lipid levels in WT mice will result in

improvements in whole-body insulin sensitivity. AccDKI mice, however, will not exhibit any improvements in insulin sensitivity with metformin treatment.

In Chapter 3, we explore the effects of exercise-induced activation of AMPK on insulin sensitivity and fatty acid metabolism in AccDKI and WT mice fed a high-fat diet. We hypothesize that AMPK activation by exercise will lead to reductions in liver lipid levels in WT mice but not AccDKI mice, leading to improvements in whole-body insulin sensitivity in WT mice only.

In Chapter 4, we explore the effects of novel AMPK activator, R419, on insulin sensitivity in AMPKβ1β2-MKO (AMPK-MKO) and WT mice fed a high-fat diet. Since R419 activates AMPK in myotubes and skeletal muscle, we hypothesize that R419 will lead to improvements in insulin sensitivity and exercise capacity in only WT mice and the same effects will not occur in AMPK-MKO mice.

CHAPTER TWO

Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin

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This manuscript examines the effects of chronic metformin treatment in obese mice. Metformin inhibits mitochondrial complex I causing an increase in AMP, which then activates AMPK. Clinical studies have demonstrated that metformin can improve hepatic insulin sensitivity and lower blood glucose in obese patients with type 2 diabetes, however, the mechanism by which metformin facilitates thse beneficial effects are unclear. To examine the effects of metformin on fatty acid metabolism and insulin sensitivity, we chronically treated high-fat diet-induced obese mice with knock-in mutations on the AMPK phosphorylation sites of ACC1 (Ser79) and ACC2 (Ser212) with metformin (50 mg/kg intraperitoneally). We show that obese AccDKI mice are resistant to the lipid lowering and insulin sensitizing effects of chronic metformin. Therefore, our data suggest that metformin treatment improves insulin resistance by reducing *de novo* lipogenesis via AMPK activation and subsequent phosphorylation of ACC.

M.D.F., S.G., B.E.K. and G.R.S. designed the study. M.D.F., S.G., K.M., S.S., R.J.F. and R.P. performed in vivo experiments. M.D.F., S.G. and J.D.S. performed primary hepatocyte experiments. S.G., Z.-P.C. performed Acc activity assays and M.O. performed mass spectrometry experiments. H.M.O. performed fatty acid oxidation in isolated skeletal muscle. T.P. and J.R.B.D. measured tissue malonyl-CoA content. D.G.H. contributed Acc antibodies for activity assays and helpful comments regarding the manuscript. B.J.v.D., S.L.M., B.E.K. and G.R.S. were involved in generating the knock-in mice. M.D.F. and G.R.S. wrote the manuscript.
Experiments that I was involved with include: Figure 1 g,h,i,j; Figure 2 a,b,c,d,e,f,g,j; Figure 3 e,f,g; Figure 4 a,b,c,d; Suppl Figure 2 i,j; Suppl. Figure 3 c,e; Suppl. Figure 4 a,b,c,d,g; Suppl. Figure 5 a,c,f,g,i; Suppl. Figure 6 a,b,d,e,f,g.

Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin

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The obesity epidemic has led to an increased incidence of nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes. AMP-activated protein kinase (Ampk) regulates energy homeostasis and is activated by cellular stress, hormones and the widely prescribed type 2 diabetes drug metformin[1,2]. Ampk phosphorylates mouse acetyl-CoA carboxylase 1 (Acc1; refs. 3,4) at Ser79 and Acc2 at Ser212, inhibiting the conversion of acetyl-CoA to malonyl-CoA. The latter metabolite is a precursor in fatty acid synthesis[5] and an allosteric inhibitor of fatty acid transport into mitochondria for oxidation[6]. To test the physiological impact of these phosphorylation events, we generated mice with alanine knock-in mutations in both Acc1 (at Ser79) and Acc2 (at Ser212) (Acc double knock-in, AccDKI). Compared to wild-type mice, these mice have elevated lipogenesis and lower fatty acid oxidation, which contribute to the progression of insulin resistance, glucose intolerance and NAFLD, but not obesity. Notably, AccDKI mice made obese by high-fat feeding are refractory to the lipid-lowering and insulin-sensitizing effects of metformin. These findings establish that inhibitory phosphorylation of Acc by Ampk is essential for the control of lipid metabolism and, in the setting of obesity, for metformin-induced improvements in insulin action.

Genetic disruption of Acc1 (refs. 7,8) or Acc2 (refs. 9–12) has yielded conflicting results as to the role of these enzymes in controlling fatty acid metabolism. The Ampk-mediated phosphorylation of Acc1 at Ser79 (Acc1 Ser79, equivalent to Acc2 Ser212) inhibits catalytic activity in cell-free systems[13]. To test the importance of Ampk signaling to Acc *in vivo*, we generated Acc1-S79A and Acc2-S212A knock-in mice and intercrossed these strains to generate AccDKI mice (**Supplementary Fig. 1a**,**b**). We examined Ampk-mediated phosphorylation of liver Acc1 Ser79 and Acc2 Ser212 by mass spectrometry and confirmed the absence of phosphorylation at these sites in the AccDKI but not the wild-type (WT) mice (**Fig. 1a** and **Supplementary Fig. 1c**). We observed no change in baseline Ampk Thr172 phosphorylation in livers from all three lines and no change in the expression of either Acc isoform (**Fig. 1a**). The activities of Acc1 and Acc2 were elevated in AccDKI mice (**Fig. 1b**,**c**) compared to WT controls, consistent with Ampk phosphorylation negatively regulating Acc1 and Acc2 enzyme activity *in vivo*. Furthermore, dephosphorylation of liver Acc1 using lambda phosphatase increased enzyme activity in liver from WT but not AccDKI mice (**Supplementary Fig. 2a**), indicating that Ser79 is the main regulatory site for Acc1 activity. Both WT and AccDKI enzymes remained sensitive to citrate activation, confirming that other mechanisms of Acc regulation remained intact in the AccDKI livers.

Liver malonyl-CoA content is dependent on Acc activity for synthesis and on malonyl-CoA decarboxylase activity for degradation. AccDKI mice had elevated liver malonyl-CoA in the fed state compared to WT control mice (**Fig. 1d**), but this did not result in compensatory upregulation of malonyl-CoA decarboxylase transcript level (**Supplementary Fig. 2b**). Hepatocytes from AccDKI mice had higher *de novo* lipogenesis (**Fig. 1e**) and lower fatty acid oxidation (**Fig. 1f**) compared to those from WT controls. Consistent with this, AccDKI mice also had higher hepatic *de novo* lipogenesis *in vivo* (**Supplementary Fig. 2c**) than WT mice. In contrast, single mutations in Acc1 or Acc2 had minimal changes in these parameters, indicating redundancy between Acc isoforms (**Supplementary Fig. 2d–f**), which is consistent with a previous siRNA knockdown study[14].

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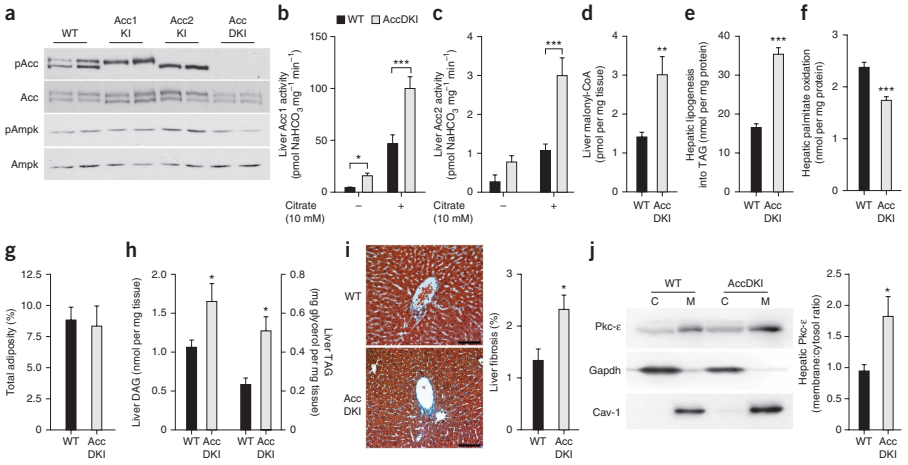

Figure 1 Acc1 Ser79 and Acc2 Ser212 are essential for inhibiting enzyme activity and regulating liver fatty acid metabolism. (**a**) Representative western blot of Ampk-α Thr172, Acc1 Ser79 (bottom band) and Acc2 Ser212 (top band) phosphorylation in liver of WT, Acc1KI, Acc2KI and AccDKI mice. pAcc, phosphorylated Acc. (**b**,**c**) Acc1 (**b**) and Acc2 (**c**) activity with and without citrate (10 mM) in WT and AccDKI liver ($n = 5$ WT and $n = 6$ AccDKI). (**d**) Liver malonyl-CoA abundance in the fed state ($n = 8$). (**e**,**f**) The incorporation of [^{3}H]acetate into TAG as a measure of *de novo* lipogenesis (**e**) and [^{14}C]palmitate oxidation (**f**) in primary hepatocytes ($n = 3$, from at least three separate experiments). (**g**) Total adiposity in chow-fed WT and AccDKI mice ($n = 10$ WT and $n = 14$ AccDKI). (**h**) Liver DAG and TAG ($n = 6$ WT and $n = 8$ AccDKI). (**i**) Histological representation (left) and quantification (right) of collagen staining in liver sections ($n = 6$). Scale bars, 100 μm. (**j**) Activation of liver Pkc-ε as demonstrated by membrane association ($n = 7$). Data are expressed as means ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ relative to WT, as determined by analysis of variance (ANOVA) and Bonferonni *post hoc* test or Student's *t*-test. For Pkc activation, Gapdh and caveolin-1 (Cav-1) were used for cytosolic (C) and membrane (M) normalization, respectively, and blots shown are from duplicate gels.

Skeletal muscle is the major tissue contributing to the basal metabolic rate, and Acc2 and malonyl-CoA have been shown to be important in regulating skeletal muscle fatty acid oxidation in some[9,11] but not all[10,12] studies. We found that relative to WT controls, malonyl-CoA was higher in skeletal muscle of AccDKI mice (**Supplementary Fig. 2g**), whereas fatty acid oxidation was slightly lower (**Supplementary Fig. 2h**). These data indicate that liver and skeletal muscle malonyl-CoA content and fatty acid metabolism are sensitive to the regulatory phosphorylation of Acc1 at Ser79 and Acc2 at Ser212.

We examined the phenotype of AccDKI mice fed a standard chow diet. Growth curves (data not shown) and adiposity were similar (**Fig. 1g**), but liver (**Fig. 1h**) and skeletal muscle (**Supplementary Fig. 2i**) diacylglycerol (DAG) and triacylglycerol (TAG) levels were elevated in AccDKI compared to WT mice. There were no differences in ceramide content in either tissue (data not shown). Elevated hepatic lipid content in AccDKI mice was associated with clinical signs of NAFLD, including an increased level of fibrosis (**Fig. 1i**) and a slightly elevated serum ratio of alanine aminotransferase to aspartate aminotransferase (**Supplementary Fig. 2j**) compared to WT controls. Pathological accumulation of DAG has been shown to activate atypical isoforms of protein kinase C (Pkc)[15], specifically Pkc-ε and Pkc-δ in liver[16] and Pkc-θ in skeletal muscle[17], which have been shown to interfere with canonical insulin signaling. Consistent with this, AccDKI mice had greater amounts of membrane-associated (**Fig. 1j**) and phosphorylated Pkc-ε (**Supplementary Fig. 3a**) in liver and Pkc-θ in skeletal muscle (**Supplementary Fig. 3b**) compared to control animals, whereas amounts of membrane-associated Pkc-δ did not differ between AccDKI and WT mice (data not shown). These results demonstrate that Acc Ser79 and Ser212 phosphorylation play an essential part in preventing ectopic lipid accumulation independent of body mass or adiposity.

The storage of excess lipid in insulin-sensitive organs such as liver and skeletal muscle is strongly associated with insulin resistance[15,18]. We found that AccDKI mice were hyperglycemic (**Fig. 2a**), hyperinsulinemic (**Fig. 2b**) and also glucose (**Fig. 2c**) and insulin intolerant (**Fig. 2d**) compared to WT controls. Hyperinsulinemic-euglycemic clamp experiments (**Supplementary Fig. 3c**) revealed that AccDKI mice had a lower glucose infusion rate (GIR) (**Fig. 2e**), a lower glucose disposal rate (GDR) (**Fig. 2e**), elevated hepatic glucose production (HGP) (**Fig. 2f**) and a lower suppression of HGP by insulin (**Fig. 2g**) compared to WT controls. Further, livers from AccDKI mice had reduced Akt kinase (Ser473) and FoxO1 transcription factor (Ser253) phosphorylation (**Fig. 2h**,**i**) and higher gluconeogenic gene expression at the completion of the clamp (**Fig. 2j**) compared to WT controls. In addition, we found that c-Jun N-terminal kinase (Jnk), which also inhibits canonical insulin signaling[19], was unchanged in livers from chow-fed AccDKI mice (**Supplementary Fig. 3d**). We observed a trend toward decreased 2-deoxyglucose (2-DG) uptake into skeletal muscle of AccDKI mice during the clamp (**Supplementary Fig. 3e**) and a marked reduction in muscle Akt (Ser473) and FoxO1 (Ser253) phosphorylation (**Supplementary Fig. 3f**,**g**) at the completion of the

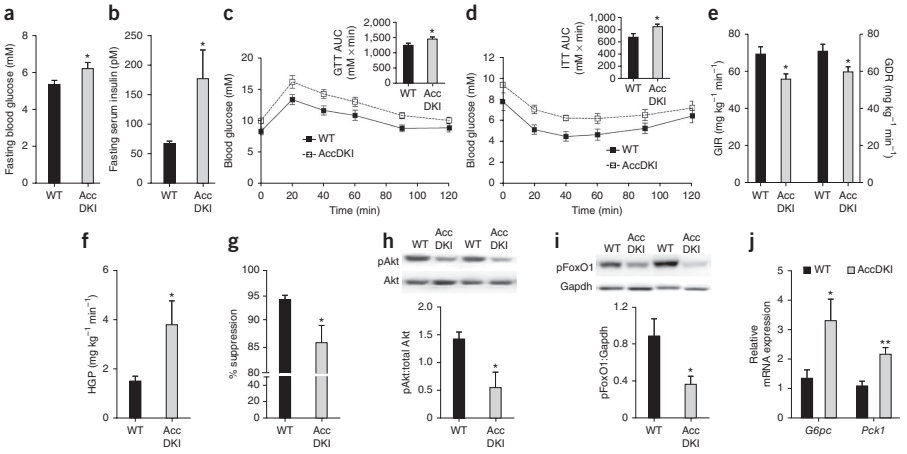

Figure 2 AccDKI mice fed a control diet are glucose intolerant and have hepatic insulin resistance. (**a**–**d**) Fasting blood glucose (**a**), fasting serum insulin levels (**b**), glucose tolerance test (GTT) (2 g per kg body weight) (**c**) and insulin tolerance test (ITT) (0.6 U per kg body weight) (**d**) ($n = 10$ WT and $n = 14$ AccDKI) in WT and AccDKI mice. AUC, area under the curve. (**e**–**g**) Hyperinsulinemic-euglycemic clamp results: GIR and GDR (**e**), HGP (**f**) and suppression of hepatic glucose production (**g**) ($n = 7$ WT and $n = 8$ AccDKI). (**h**–**j**) Liver Akt (Ser473) phosphorylation (**h**), liver FoxO1 (Ser253) phosphorylation (**i**) and gluconeogenic gene expression (*G6pc* and *Pck1*) (**j**) in the liver at the completion of the clamp ($n = 7$ WT and $n = 8$ AccDKI). Data are expressed as means ± s.e.m. $^*P < 0.05$ and $^{**}P < 0.01$ relative to WT, as determined by Student's *t*-test. Relative gene expression was normalized to *Actb*, and duplicate gels were run for quantification of total Akt and Gapdh.

clamp compared to WT control mice. Furthermore, insulin resistance in AccDKI mice was independent of changes in liver or adipose tissue macrophage accumulation, inflammatory cytokine gene expression or protein content (**Supplementary Fig. 4a–c**) or differences in circulating free fatty acids (**Supplementary Fig. 4d**). These results indicate that Acc phosphorylation is required to maintain insulin sensitivity in lean healthy mice. Notably, mice with complete deletions of Ampk isoforms in skeletal muscle[20] or liver[21] have normal lipid levels and insulin sensitivity, suggesting that in these models, there may be alterations in compensatory pathways that are important for controlling fatty acid metabolism.

Over 120 million people are prescribed metformin for the management of type 2 diabetes[22]. As metformin indirectly activates Ampk, it was initially thought that Ampk mediates metformin's therapeutic actions[23]. However, acute inhibition of gluconeogenesis by metformin is independent of Ampk[21] and involves inhibition of glucagon signaling through protein kinase A (Pka)[24]. Nevertheless, the ability of metformin to lower blood glucose in obese individuals with type 2 diabetes involves chronic enhancement in insulin sensitivity[25–29]. We found that acute metformin treatment activated hepatic Ampk in both genotypes, but this was only associated with increased Acc phosphorylation (**Fig. 3a**) and reduced malonyl-CoA levels (**Fig. 3b**) in WT mice. Metformin reduced *de novo* lipogenesis in hepatocytes from WT mice, and this suppressive effect was equivalent to that caused by Ampk-β1–specific activation using A-769662 (ref. 30) (**Fig. 3c**). Notably, the metformin-induced inhibition of hepatic lipogenesis seen upon Ampk activation was entirely mediated by Ampk phosphorylation of Acc, as metformin and A-769662 were ineffective at suppressing lipogenesis in AccDKI or Ampk-β1–deficient hepatocytes (**Fig. 3c**). However, unlike A-769662, metformin did not increase fatty acid oxidation in either genotype (**Supplementary Fig. 4e**). These data demonstrate that the effects of metformin on lipogenesis are specific to Ampk and indicate that although Ampk may inhibit multiple targets in this pathway, including sterol regulatory element binding protein-1c (ref. 31) and expression of fatty acid synthase[32], the primary regulation of lipogenesis is dependent on the phosphorylation of both Acc1 and Acc2. In contrast, lipogenesis was inhibited by metformin in hepatocytes from both WT and Acc1 knock-in (Acc1KI) mice (**Supplementary Fig. 4f**), and *in vivo*, a lipid-lowering effect was demonstrated in high-fat diet (HFD)-fed WT and Acc1KI animals treated with metformin (**Supplementary Fig. 4g**).

In humans, therapeutic doses of metformin (0.5–3 g per day)[27] result in plasma concentrations ranging from 10 to 25 μM (refs. 33,34). In rodents, the administration of 50 mg per kg body weight of metformin has been shown to elicit plasma concentrations of 29 μM (ref. 35). We therefore treated HFD-fed WT and AccDKI mice with a daily dose of metformin at 50 mg per kg body weight for 6 weeks. In contrast to chow-fed mice, HFD-fed AccDKI mice showed no differences in any metabolic parameters compared to HFD-fed WT animals (**Fig. 3** and **Supplementary Figs. 5–7**). This indicates that diet-induced obesity overwhelms the effect of signaling by endogenous Ampk to Acc unless an external Ampk stimulus is provided.

Metformin has been shown to have positive effects on fatty liver in some but not all clinical trials[25]. However, rodent studies with metformin have demonstrated a clear lipid-lowering effect[23,30], which suggests the need for more robust analyses or the development of more reliable biomarkers in human studies[25,36]. Notably, *in vivo*

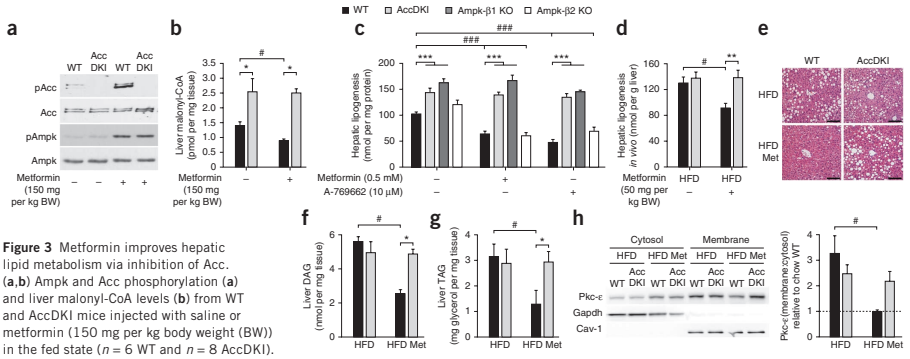

Figure 3 Metformin improves hepatic lipid metabolism via inhibition of Acc. (**a**,**b**) Ampk and Acc phosphorylation (**a**) and liver malonyl-CoA levels (**b**) from WT and AccDKI mice injected with saline or metformin (150 mg per kg body weight (BW)) in the fed state ($n = 6$ WT and $n = 8$ AccDKI). (**c**) Incorporation of [^{3}H]acetate into the total lipid fraction (*de novo* lipogenesis) in primary hepatocytes ($n = 3$ from at least three separate experiments). KO, knockout. (**d**) *In vivo* incorporation of [^{3}H]acetate into total liver lipid (*de novo* lipogenesis) in HFD-fed WT and AccDKI mice treated with vehicle or metformin (50 mg per kg body weight) ($n = 11$ for vehicle and $n = 6$ for metformin). (**e**–**g**) Representative staining (H&E) of hepatic sections (**e**) (scale bars, 100 μm) and determination of hepatic DAG (**f**) and TAG (**g**) ($n = 7$ WT and $n = 8$ AccDKI) from WT and AccDKI mice fed a HFD for 12 weeks, with or without concurrent metformin (Met) (50 mg per kg body weight per day) starting after 6 weeks of HFD (HFD Met). (**h**) Activation of hepatic Pkc-ε, shown as the ratio of membrane to cytosolic expression and expressed relative to chow WT control ($n = 7$) (cytosol normalized to Gapdh and membrane normalized to caveolin-1 (Cav-1); blots shown are from duplicate gels). Data are expressed as means ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to WT control and $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.01$ are differences between treatment, as calculated by two-way ANOVA and Bonferonni *post hoc* test.

lipogenesis was similar between HFD-fed WT and AccDKI mice (**Fig. 3d**), and an acute dose of metformin (50 mg per kg body weight) suppressed lipogenesis *in vivo* by ~35% in WT livers yet was completely ineffective in AccDKI mice (**Fig. 3d**). We next assessed the metabolic effects of chronic metformin treatment and found that, independent of change in weight or adiposity (**Supplementary Fig. 5c**), hepatic lipid content was reduced in WT mice, an effect completely absent in AccDKI mice (**Fig. 3e**–**g**). Reductions in hepatic DAG were accompanied by decreased membrane-associated (**Fig. 3h**) and Ser729-phosphorylated Pkc-ε (**Supplementary Fig. 5d**) and lower Jnk activation (**Supplementary Fig. 5e**) in metformin-treated WT but not AccDKI mice.

In addition to reducing hepatic lipid content, chronic metformin treatment of HFD-fed WT but not AccDKI mice was associated with lowered fasting blood glucose (**Fig. 4a**), a trend toward lowered serum insulin levels (**Supplementary Fig. 5f**) and improved glucose tolerance (**Supplementary Fig. 5g**) and insulin sensitivity (**Fig. 4b**). Metformin-induced suppression of cAMP and glucagon-dependent hepatic glucose output is independent of Ampk[21,24], and in hepatocytes from WT, AccDKI and Ampk-β1–deficient mice, metformin was effective at suppressing cAMP-stimulated glucose production (**Supplementary Fig. 5h**). Metformin was also able to acutely lower circulating glucose levels in both chow-fed and obese HFD-fed WT and AccDKI mice, as shown by metformin tolerance tests (200 mg per kg body weight[37]) (**Supplementary Fig. 5i**). However, at the dose that was used for our chronic treatments (50 mg per kg body weight), glucose levels were unaltered (**Supplementary Fig. 5j**), strongly suggesting that metabolic differences following chronic metformin treatment between AccDKI and WT mice were primarily the result of differential regulation of insulin sensitivity rather than acute effects on glucose lowering.

In hyperinsulinemic-euglycemic clamp experiments, chronic metformin treatment improved GDR (**Supplementary Fig. 6a**) and skeletal muscle 2-DG uptake in both WT and AccDKI mice (**Supplementary Fig. 6b**,**c**). This is consistent with an Ampk-Pkc–dependent pathway controlling metformin-induced skeletal muscle glucose uptake[38]. In contrast, chronic metformin treatment increased GIR (**Supplementary Fig. 6d**–**g**), decreased HGP (**Fig. 4c**) and increased suppression of HGP by insulin (**Fig. 4d**) in WT but not AccDKI mice. Enhanced Akt (Ser473) and FoxO1 (Ser253) phosphorylation (**Supplementary Fig. 7a**,**b**) and reduced gluconeogenic gene expression (**Supplementary Fig. 7c**) at the completion of the clamp in WT but not in AccDKI mice provides further evidence of metformin-induced improvements in hepatic insulin sensitivity. We demonstrated that metformin improved insulin sensitivity in a liver cell-autonomous manner in a cellular model of palmitate-induced insulin resistance. In particular, hepatocytes made insulin resistant by chronic treatment (18 h) with the saturated fatty acid palmitate were treated with metformin, which improved insulin-stimulated phosphorylation of Akt Ser473 and FoxO1 Ser253 (**Fig. 4e**), insulin-induced suppression of gluconeogenic gene expression (**Fig. 4f**) and insulin-induced suppression of hepatic glucose production (**Fig. 4g**) in hepatocytes from WT but not AccDKI mice. Notably, the beneficial metabolic effects of specific Ampk activation by A-769662 were completely abrogated in AccDKI hepatocytes and *in vivo* (**Supplementary Fig. 8a**–**d**), which corroborates the fundamental importance of Ampk-Acc signaling for hepatic lipid metabolism and insulin sensitivity.

Metformin remains the primary therapeutic option for the treatment of type 2 diabetes, although the precise mechanisms by which it confers its beneficial effects are incompletely understood. Glucagon-driven hepatic gluconeogenesis maintains glycemia during states of fasting, and this is poorly regulated in patients with type 2 diabetes. Recently, it has been shown that metformin counters this program by inhibiting glucagon-stimulated cAMP production, thereby reducing Pka activity and glucagon-stimulated glucose output from

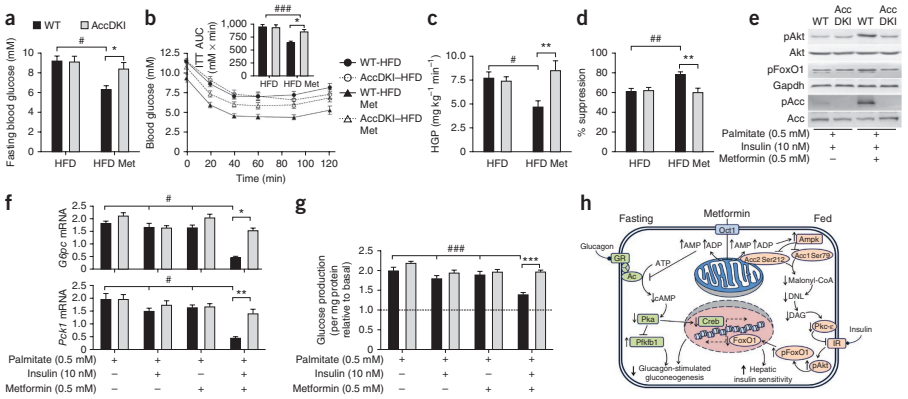

Figure 4 HFD-fed AccDKI mice are insensitive to metformin-induced improvements in liver insulin sensitivity. (**a**–**d**) WT and AccDKI mice fed a HFD for 6 weeks were given daily metformin (50 mg per kg body weight) for an additional 6 weeks (HFD Met). Fasting blood glucose (**a**) and insulin tolerance test (1 U per kg body weight) (**b**) ($n = 10$ HFD-fed WT and AccDKI; $n = 12$ WT and $n = 16$ AccDKI–HFD Met). The effect of metformin treatment on HGP (**c**) and suppression of HGP by insulin (**d**) ($n = 7$ WT and $n = 9$ AccDKI). (**e**,**f**) Akt (Ser473) and FoxO1 (Ser253) phosphorylation (**e**) and *G6pc* and *Pck1* expression (**f**) in isolated hepatocytes treated with chronic palmitate (18 h) and stimulated with insulin, where gene expression is shown relative to the WT condition without palmitate. (**g**) Hepatic glucose production, following chronic (18 h) exposure to palmitate (0.5 mM) in the presence or absence of metformin (0.5 mM), then in response to Bt_2-cAMP (100 μM) and insulin (10 nM) for 4 h, in the absence of acute metformin. ($n = 3$, from at least three separate experiments). Hatched line represents control hepatocytes not stimulated with Bt_2-cAMP for glucose production. (**h**) Schematic representation of metformin's therapeutic effects on hepatic action during differential nutrient and hormonal programs. Oct1, organic cation transporter 1; GR, glucagon receptor; Ac, adenylate cyclase; DNL, *de novo* lipogenesis; Creb, cAMP response element–binding protein; Pfkfb1, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1; IR, insulin receptor. Data are expressed as means ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ represent differences between genotype and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ are differences between treatment, as calculated by two-way ANOVA and Bonferonni *post hoc* test.

the liver[24]. We confirmed that this mechanism also operates in both WT and AccDKI mice in response to high concentrations of metformin (**Supplementary Fig. 8e**). An important distinction between this previous work[24] and our current study is their focus on the fasting, or glucagon-specific, actions of metformin, which occur in the absence of insulin stimulation. The ability of insulin to suppress hepatic gluconeogenesis and to promote the efficient uptake of glucose in the periphery is fundamental and dramatically decreased in insulin resistance and type 2 diabetes.

The insulin-sensitizing effects of metformin have been well documented[25,28,29], but mechanistic insight has been lacking. Our data provide evidence that in the setting of obesity and insulin resistance, chronic metformin treatment (at a clinical dose) reduces hepatic lipogenesis and lipid accumulation by activation of Ampk and consequent inhibition of both Acc1 and Acc2. This lipid-lowering effect then alleviates obesity-induced insulin resistance. Notwithstanding metformin inhibition of glucagon-dependent gluconeogenesis, we provide evidence for a parallel mechanism, whereby chronic metformin treatment increases insulin sensitivity through alterations in hepatic lipid homeostasis (**Fig. 4h**).

Since the initial discovery that Ampk directly phosphorylates Acc1, the site-specific phosphorylation of Acc1 and Acc2 has been used as a surrogate marker for Ampk signaling in hundreds of studies. Currently, more than 30 other substrates of Ampk have been identified in multiple metabolic pathways. Through genetic targeting of Acc1 Ser79 and Acc2 Ser212 and the generation of knock-in mice, we show that phosphorylation and inhibition of these two Ampk substrates is critical for maintaining lipid metabolism and insulin sensitivity. Moreover, Acc phosphorylation by Ampk also underpins the insulin-sensitizing effects of metformin.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.D.F., S.G., B.E.K. and G.R.S. designed the study. M.D.F., S.G., K.M., S.S., R.J.F. and R.P. performed *in vivo* experiments. M.D.F., S.G. and J.D.S. performed primary hepatocyte experiments. S.G., Z.-P.C. performed Acc activity assays and M.O. performed mass spectrometry experiments. H.M.O. performed fatty acid oxidation in isolated skeletal muscle. T.P. and J.R.B.D. measured tissue malonyl-CoA content. D.G.H. contributed Acc antibodies for activity assays and helpful comments

regarding the manuscript. B.J.v.D., S.L.M., B.E.K. and G.R.S. were involved in generating the knock-in mice. M.D.F. and G.R.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

1. Kahn, B.B., Alquier, T., Carling, D. & Hardie, D.G. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* **1**, 15–25 (2005).
2. Long, Y.C. & Zierath, J.R. AMP-activated protein kinase signaling in metabolic regulation. *J. Clin. Invest.* **116**, 1776–1783 (2006).
3. Carlson, C.A. & Kim, K.H. Regulation of hepatic acetyl coenzyme A carboxylase by phosphorylation and dephosphorylation. *J. Biol. Chem.* **248**, 378–380 (1973).
4. Carling, D., Zammit, V.A. & Hardie, D.G. A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. *FEBS Lett.* **223**, 217–222 (1987).
5. Wakil, S.J., Stoops, J.K. & Joshi, V.C. Fatty acid synthesis and its regulation. *Annu. Rev. Biochem.* **52**, 537–579 (1983).
6. McGarry, J.D., Leatherman, G.F. & Foster, D.W. Carnitine palmitoyltransferase I. The site of inhibition of hepatic fatty acid oxidation by malonyl-CoA. *J. Biol. Chem.* **253**, 4128–4136 (1978).
7. Harada, N. *et al.* Hepatic *de novo* lipogenesis is present in liver-specific ACC1-deficient mice. *Mol. Cell Biol.* **27**, 1881–1888 (2007).
8. Mao, J. *et al.* Liver-specific deletion of acetyl-CoA carboxylase 1 reduces hepatic triglyceride accumulation without affecting glucose homeostasis. *Proc. Natl. Acad. Sci. USA* **103**, 8552–8557 (2006).
9. Abu-Elheiga, L., Matzuk, M.M., Abo-Hashema, K.A. & Wakil, S.J. Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. *Science* **291**, 2613–2616 (2001).
10. Hoehn, K.L. *et al.* Acute or chronic upregulation of mitochondrial fatty acid oxidation has no net effect on whole-body energy expenditure or adiposity. *Cell Metab.* **11**, 70–76 (2010).
11. Olson, D.P., Pulinilkunnil, T., Cline, G.W., Shulman, G.I. & Lowell, B.B. Gene knockout of Acc2 has little effect on body weight, fat mass, or food intake. *Proc. Natl. Acad. Sci. USA* **107**, 7598–7603 (2010).
12. Choi, C.S. *et al.* Continuous fat oxidation in acetyl-CoA carboxylase 2 knockout mice increases total energy expenditure, reduces fat mass, and improves insulin sensitivity. *Proc. Natl. Acad. Sci. USA* **104**, 16480–16485 (2007).
13. Munday, M.R., Campbell, D.G., Carling, D. & Hardie, D.G. Identification by amino acid sequencing of three major regulatory phosphorylation sites on rat acetyl-CoA carboxylase. *Eur. J. Biochem.* **175**, 331–338 (1988).
14. Savage, D.B. *et al.* Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. *J. Clin. Invest.* **116**, 817–824 (2006).
15. Erion, D.M. & Shulman, G.I. Diacylglycerol-mediated insulin resistance. *Nat. Med.* **16**, 400–402 (2010).
16. Samuel, V.T. *et al.* Inhibition of protein kinase Cepsilon prevents hepatic insulin resistance in nonalcoholic fatty liver disease. *J. Clin. Invest.* **117**, 739–745 (2007).
17. Yu, C. *et al.* Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J. Biol. Chem.* **277**, 50230–50236 (2002).
18. Chibalin, A.V. *et al.* Downregulation of diacylglycerol kinase δ contributes to hyperglycemia-induced insulin resistance. *Cell* **132**, 375–386 (2008).
19. Hirosumi, J. *et al.* A central role for JNK in obesity and insulin resistance. *Nature* **420**, 333–336 (2002).
20. O'Neill, H.M. *et al.* AMP-activated protein kinase (AMPK) β1β2 muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. *Proc. Natl. Acad. Sci. USA* **108**, 16092–16097 (2011).
21. Foretz, M. *et al.* Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J. Clin. Invest.* **120**, 2355–2369 (2010).
22. Viollet, B. *et al.* Cellular and molecular mechanisms of metformin: an overview. *Clin. Sci. (Lond.)* **122**, 253–270 (2012).
23. Zhou, G. *et al.* Role of AMP-activated protein kinase in mechanism of metformin action. *J. Clin. Invest.* **108**, 1167–1174 (2001).
24. Miller, R.A. *et al.* Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature* **494**, 256–260 (2013).
25. Musso, G., Cassader, M., Rosina, F. & Gambino, R. Impact of current treatments on liver disease, glucose metabolism and cardiovascular risk in non-alcoholic fatty liver disease (NAFLD): a systematic review and meta-analysis of randomised trials. *Diabetologia* **55**, 885–904 (2012).
26. Bailey, C.J. & Mynett, K.J. Insulin requirement for the antihyperglycaemic effect of metformin. *Br. J. Pharmacol.* **111**, 793–796 (1994).
27. Bailey, C.J. & Turner, R.C. Metformin. *N. Engl. J. Med.* **334**, 574–579 (1996).
28. Gómez-Sámano, M.Á. *et al.* Metformin and improvement of the hepatic insulin resistance index independent of anthropometric changes. *Endocr. Pract.* **18**, 8–16 (2012).
29. Natali, A. & Ferrannini, E. Effects of metformin and thiazolidinediones on suppression of hepatic glucose production and stimulation of glucose uptake in type 2 diabetes: a systematic review. *Diabetologia* **49**, 434–441 (2006).
30. Cool, B. *et al.* Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metab.* **3**, 403–416 (2006).
31. Li, Y. *et al.* AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell Metab.* **13**, 376–388 (2011).
32. Foretz, M., Carling, D., Guichard, C., Ferre, P. & Foufelle, F. AMP-activated protein kinase inhibits the glucose-activated expression of fatty acid synthase gene in rat hepatocytes. *J. Biol. Chem.* **273**, 14767–14771 (1998).
33. Lalau, J.D., Lemaire-Hurtel, A.S. & Lacroix, C. Establishment of a database of metformin plasma concentrations and erythrocyte levels in normal and emergency situations. *Clin. Drug Investig.* **31**, 435–438 (2011).
34. Owen, M.R., Doran, E. & Halestrap, A.P. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem. J.* **348**, 607–614 (2000).
35. Wilcock, C. & Bailey, C.J. Accumulation of metformin by tissues of the normal and diabetic mouse. *Xenobiotica* **24**, 49–57 (1994).
36. Clark, J.M., Brancati, F.L. & Diehl, A.M. Nonalcoholic fatty liver disease. *Gastroenterology* **122**, 1649–1657 (2002).
37. He, L. *et al.* Metformin and insulin suppress hepatic gluconeogenesis through phosphorylation of CREB binding protein. *Cell* **137**, 635–646 (2009).
38. Turban, S. *et al.* Defining the contribution of AMP-activated protein kinase (AMPK) and protein kinase C (PKC) in regulation of glucose uptake by metformin in skeletal muscle cells. *J. Biol. Chem.* **287**, 20088–20099 (2012).

ONLINE METHODS

Animals. Both Acc1-S79A KI and Acc2-S212A KI mice were generated by OzGene Pty (Perth, Australia). The targeting strategy is summarized in **Supplementary Figure 1**. The generation of Ampk-β1–deficient and Ampk-β2–deficient mice has previously been described[39,40]. All mice used in the study were bred on a C57BL/6 background and from heterozygous intercrosses. Male mice were used for all studies and housed in specific-pathogen–free microisolators and maintained on a 12-h light-dark cycle with lights on at 7:00 a.m. Mice were maintained on either a chow diet (17% kcal from fat; Diet 8640, Harlan Teklad, Madison, WI) or HFD (45% kcal from fat; D12451, Research Diets; New Brunswick, NJ) starting at 6 weeks of age for 12 weeks. For HFD-metformin experiments, mice received 6 weeks of daily intraperitoneal injections of metformin (50 mg per kg body weight) starting after 6 weeks of the HFD. Fasting and fed blood samples were collected for serum analyses through submandibular bleeding. The McMaster University (Hamilton, Canada) Animal Ethics Research Board and St. Vincent's Hospital (Melbourne, Australia) Animal Ethics Committee approved all experimental protocols.

Enzymatic activity assays. Acc activity in liver was measured by $^{14}CO_2$ fixation into acid-stable products. Acc1 or Acc2 protein was immunoprecipitated from 2 mg of tissue homogenates using Acc1- or Acc2-specific antibodies. Acc1- and Acc2-specific antibodies were generated by immunizing sheep with synthetic peptides coupled to keyhole limpet hemocyanin (CDEPSPLAKTLELNQ (rat Acc1 (1–15 Cys^1) and CEDKKQAPIKRQLMT (rat Acc2 (145–159 Cys^{145})) and purifying antibodies from the resulting sera by affinity chromatography on immobilized peptides. Immunoprecipitates were incubated for 1.5 h at room temperature with reaction buffer containing 125 μM acetyl-CoA, 12.5 mM $NaHCO_3$ and 16.7 μCi/ml [^{14}C]$NaHCO_3$ with the indicated concentrations of citrate. The reactions were terminated by addition of HCl and dried overnight at 37 °C. Water was added to the dried sample and radioactivity measured by liquid scintillation counting. Purified Acc1 proteins from WT and Acc1KI livers were subjected to λ phosphatase (400 U in a 60-μl reaction) treatment for 25 min at 30 °C. After a wash removal of the phosphatase, a small aliquot was used for determination of Acc1 Ser79 phosphorylation by western blot analysis. The majority of the protein that remained was used to assess Acc activity, as described above.

Mass spectrometry analyses. Acc isoforms from WT and AccDKI livers were affinity-purified by streptavidin. Proteins were eluted from beads, reduced and alkylated with iodoacetamide and then precipitated with methanol/chloroform (1:1, vol/vol). The denatured proteins were digested overnight with trypsin in solution at 37 °C. Peptides were separated on a PepMap RSLC C_{18} column using a Dionex 3000 Series NCS-3500RS nano liquid chromatography system. Mass spectrometry was performed on a 5600 Triple TOF MS (AB SCIEX).

Malonyl-CoA assay. Quantification of short-chain CoA species was performed as reported previously with modification[41]. Briefly, frozen muscle and liver samples (~15–20 mg) were homogenized for 20 s in 300 μl of 6% (vol/vol) perchloric acid. After homogenization, the samples were left on ice for 10 min and then centrifuged at 12,000*g* for 5 min. The resulting supernatant (100 μl) was analyzed using a UPLC Waters Acquity System. Each sample was run at a flow rate of 0.4 ml/min through an Ascentis Express C_{18} Column, 10 cm × 2.1 mm and 2.7-μm particle size from Supelco maintained at a temperature of 40 °C. The analyte detection occurred at an absorbance of 260 nm. The mobile phase consisted of a mixture of buffer A (0.25 M NaH_2PO_4 and water) and buffer B (0.25 M NaH_2PO_4 and acetonitrile). The gradient-elution profile consisted of the following initial conditions: 2% B for 2–4 min, 25% B for 4–6 min, 40% B for 6–8 min and 100% B for 10–12 min, maintained for 15 min. All gradients were linear and peaks were acquired, integrated and analyzed using the Waters Empower Software.

Histological analyses. Tissues were fixed in formalin for at least 48 h, embedded in paraffin and H&E stained. After staining, each sample was imaged in triplicate. For determination of hepatic fibrosis, trichrome staining was used to visualize collagen. This was then quantified using the color segmentation function of Image J software (National Institutes of Health).

Western blotting, inflammation and RT-PCR. Tissues were dissected rapidly, snap-frozen in liquid nitrogen and stored at −80 °C until subsequent analyses. All primary antibodies were used at a dilution of 1:1,000. Blotting for total and phosphorylated Akt, Jnk, Ampk and Acc (antibodies all from Cell Signaling: Akt #9272, pAkt Ser473 #4058, Jnk #9252, pJNK #9251, AMPK-α #2532, pAMPK-α Thr172 #2531, Acc #3676, pAcc Ser79 #3661 and β-actin #5125) were performed as previously described[42]. Phosphorylated FoxO1 Ser253 (Cell Signaling #9461) was normalized to Gapdh (Cell Signaling #2118). For Pkc-ε phosphorylation, an antibody directed against Ser729 was used (Abcam ab134031). For membrane-associated Pkc activation, tissues were homogenized in 300 μl of buffer I (20 mM Tris·HCl, pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 250 mM sucrose and protease inhibitor mixture) and centrifuged at 100,000 × *g* for 1 h (4 °C). The supernatants containing the cytosolic fraction were removed to new tubes. Pellets were then resuspended in 300 μl of buffer II (250 mM Tris·HCl, pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 2% Triton X-100 and protease inhibitor mixture) and centrifuged at 100,000 × *g* for 1 h (4 °C) to obtain the plasma membrane fraction. Gapdh and caveolin-1 were used as markers of the cytosolic and membrane preparation purity, respectively. Pkc-ε, Pkc-θ and Pkc-δ activation (Cell signaling Pkc- ε #2683, Pkc-θ #2059 and Pkc-δ #2058) was expressed as a ratio of membrane (normalized to caveolin-1(BD Biosciences #C37120)) to cytosolic (normalized to Gapdh) localization, which was assessed from the same membrane to eliminate exposure bias. Hepatic tissues were prepared as previously described[43], and proinflammatory cytokines were determined using commercially available kits. Total RNA isolation, cDNA synthesis and quantitative RT-PCR were performed as described previously[42].

Metabolic studies. For glucose, insulin and metformin tolerance tests, mice were injected with D-glucose (2 g per kg body weight and 1 g per kg body weight for chow and HFD, respectively), human insulin (0.6 U per kg body weight and 1 U per kg body weight for chow and HFD, respectively) or 50 and 200 mg per kg body weight metformin[37] through intraperitoneal injection and blood glucose monitored at the indicated times by a small cut in the tail vein. Whole-body adiposity was assessed by computed tomography, and respiratory exchange ratio was determined using Columbus Laboratory Animal Monitoring System as previously described[42]. Hyperinsulinemic-euglycemic clamps were performed as previously described[42]. Briefly, 5 d after cannulation, only mice that lost <8% of their weight were clamped. Mice fasted 6 h were infused with a basal infusate containing D-[3-^{3}H]glucose (7.5 μCi/h, 0.12 ml/h) for 1 h to determine basal glucose disposal. An insulin infusate (10 mU insulin per kg body weight per min in 0.9% saline) containing D-[3-^{3}H]glucose (7.5 μCi/h, 0.12 ml/h) was then initiated and blood glucose monitored and titrated with 50% dextrose infused at a variable rate to achieve and maintain euglycemia. For rates of glucose uptake, [^{14}C]2-DG (10 μCi) was infused during the clamped state and tissues excised after 30 min. The rates of glucose disposal in the basal and clamped states and hepatic glucose output were calculated using the Steele equation for steady-state conditions[44]. Insulin was measured by ELISA kit, alanine aminotransferase and aspartate aminotransferase were determined using commercially available kits and TAG levels were determined by glycerol assay following saponification of the TAG fraction separated using thin-layer chromatography. Total levels of tissue DAG and ceramides were quantified using DAG kinase assay as previously described[45]. *de novo* lipogenesis was determined *in vivo* by the incorporation of [^{3}H]acetate into hepatic lipids after metformin (50 mg per kg body weight) or A-769662 (30 mg per kg body weight) injection, where saline and 5% DMSO in PBS served as vehicles, respectively. Skeletal muscle fatty acid oxidation was assessed in isolated extensor digitorum longus muscle as previously described[40]. For metformin-stimulated glucose uptake, isolated extensor digitorum longus muscle was incubated with vehicle (60 min), metformin (1 mM for 60 min), submaximal insulin (2.0 μM for 30 min) or metformin (1 mM for 60 min) plus insulin (2.0 μM for 30 min). 2-DG uptake was then measured over 20 min in the presence of insulin (2 μM) or metformin (1 mM). To measure hepatic cAMP, WT and AccDKI mice in the fed condition were

injected with vehicle, 200 or 400 mg per kg body weight metformin. After 1 h, mice received a vehicle or glucagon injection (2 mg per kg body weight). Livers were collected after 5 min and rapidly freeze-clamped[23]. Hepatic cAMP measurements were performed using an EIA from Cayman Chemicals per the manufactures instructions.

Cell culture experiments. Primary hepatocytes were isolated by collagenase perfusion[42]. [^{3}H]acetate lipogenesis and [^{14}C]palmitate oxidation was performed as described previously[39]. Briefly, for lipogenesis, [^{3}H]acetate (5 μCi/ml) was in the presence of 0.5 mM sodium acetate for 4 h. Medium was then removed and cells washed with PBS before lipid extraction for determination of incorporation into lipid fractions. For fatty acid oxidation, [^{14}C]palmitate (2 μCi/ml) was in the presence of 0.5 mM palmitate (conjugated to 2% BSA) for 4 h. Medium was removed and acidified with equal volume of 1 M acetic acid in an airtight vial. [^{14}C]CO_2 was trapped in 400 μl of 1 M benzethonium hydroxide and radioactivity determined. Cellular lipids were extracted after a PBS wash, and radioactivity of the acid soluble intermediates was determined. Total oxidation was then calculated as a function of both [^{14}C]CO_2–produced and incomplete oxidation products. For insulin signaling experiments in hepatocytes, cells were incubated in the presence or absence of 0.5 mM palmitate (conjugated to 2% BSA) and 0.5 mM metformin for 18 h (with 0.1% FBS). Cells were then stimulated with 10 nM insulin; gluconeogenic gene expression was assessed after 6 h of insulin treatment, and insulin signaling was assessed after 5 min of insulin stimulation. Hepatic glucose production was determined as previously described[22]. Briefly, cells were cultured as above in the presence of 100 nM dexamethasone. Cells were washed once with PBS and incubated in glucose-free DMEM (100 nM dexamethasone, 10 mM lactate and 1 mM pyruvate) with or without Bt_2-cAMP (100 μM) and with or without indicated doses of metformin or A-769662. For chronic treatments, palmitate-, metformin- and A-769662–supplemented medium was removed after 18 h, cells were washed and glucose-free DMEM was added to measure glucose output in the presence or absence of insulin (10 nM). Glucose in the medium after 4 h was assessed by glucose oxidase kit.

Statistical analyses. All results shown are mean ± s.e.m. Results were analyzed using a two-tailed Student's *t*-test or two-way ANOVA, where appropriate, using GraphPad Prism software. A Bonferonni *post hoc* test was used to test for significant differences revealed by the ANOVA. Significance was accepted at $P \leq 0.05$.

39. Dzamko, N. *et al.* AMPK β1 deletion reduces appetite, preventing obesity and hepatic insulin resistance. *J. Biol. Chem.* **285**, 115–122 (2010).
40. Steinberg, G.R. *et al.* Whole body deletion of AMP-activated protein kinase β2 reduces muscle AMPK activity and exercise capacity. *J. Biol. Chem.* **285**, 37198–37209 (2010).
41. Ussher, J.R. *et al.* Stimulation of glucose oxidation protects against acute myocardial infarction and reperfusion injury. *Cardiovasc. Res.* **94**, 359–369 (2012).
42. Galic, S. *et al.* Hematopoietic AMPK β1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. *J. Clin. Invest.* **121**, 4903–4915 (2011).
43. Schertzer, J.D. *et al.* NOD1 activators link innate immunity to insulin resistance. *Diabetes* **60**, 2206–2215 (2011).
44. Steele, R. Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann. NY Acad. Sci.* **82**, 420–430 (1959).
45. Preiss, J. *et al.* Quantitative measurement of *sn*-1,2-diacylglycerols present in platelets, hepatocytes, and *ras*- and *sis*-transformed normal rat kidney cells. *J. Biol. Chem.* **261**, 8597–8600 (1986).

Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin–sensitizing effects of metformin

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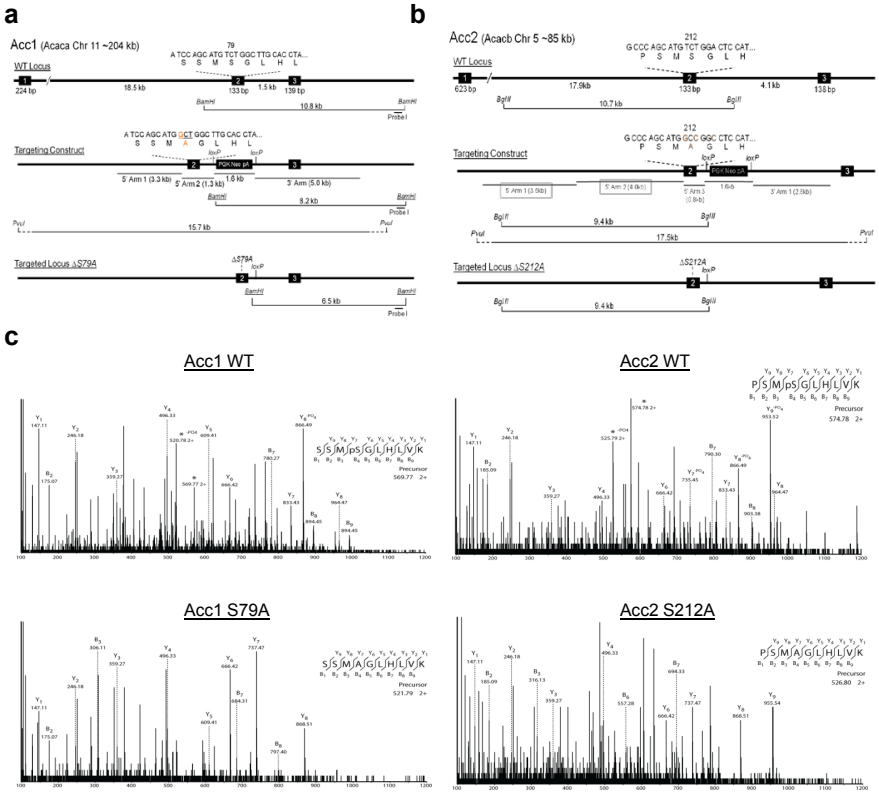
Supplementary Figure 1



Supplementary Fig. 1. Generation and confirmation of Acc1KI and Acc2KI mice. Schematic representation of the targeting strategy for (**a**) Acc1KI and (**b**) Acc2KI mice. (**c**) Summary of mass spectroscopy analyses of the Ser–Ala mutation on Acc1 and Acc2, where the precursor denotes the charge at the site of the mutation at Acc1 Ser79 and Acc2 Ser212.

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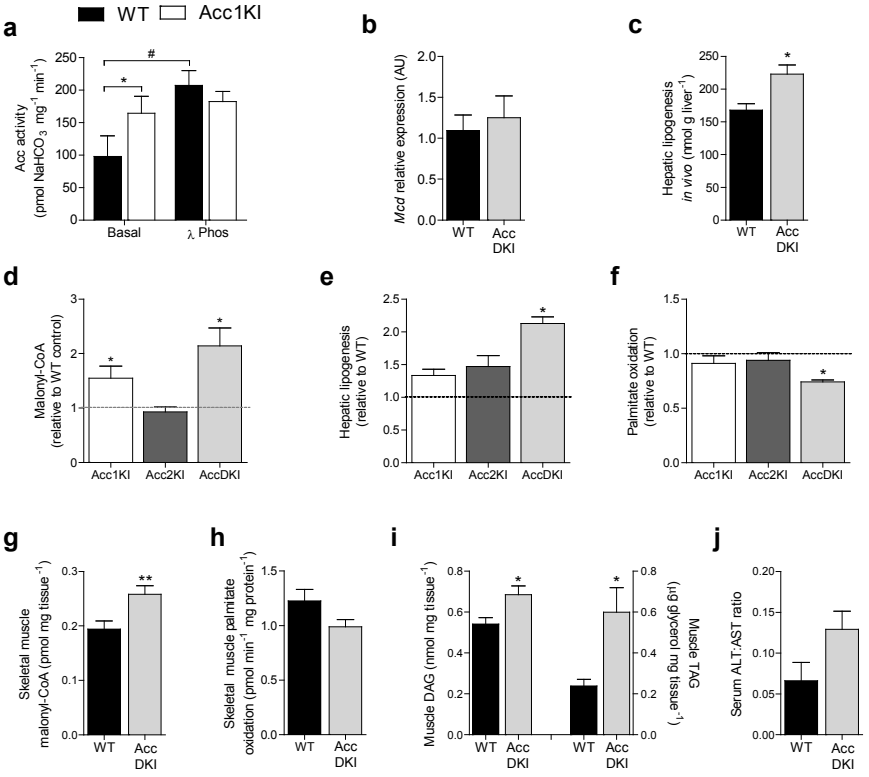
Supplementary Figure 2



Supplementary Fig. 2. Metabolic profile of Acc1KI, Acc2KI and AccDKI in liver and skeletal muscle. (**a**) Acc1 enzyme activity was assessed under basal conditions (in the absence of citrate) and after λ phosphatase treatment from WT and Acc1KI livers (n = 4). (**b**) Liver malonyl–CoA decarboxylase (*Mcd*) expression in AccDKI mice relative to WT control (n = 7–8). (**c**) The *in vivo* incorporation of [^{3}H]–acetate into total liver lipid as a measure of *de novo* lipogenesis (n = 5). (**d**) Liver malonyl–CoA levels (n = 7–8), (**e**) hepatocyte lipogenesis ([^{3}H]–acetate incorporation into TAG) and (**f**) hepatocyte [^{14}C]–palmitate oxidation from Acc1KI, Acc2KI and AccDKI mice, expressed relative to WT control (n = 3 from at least 3 separate experiments). (**g**) Skeletal muscle malonyl–CoA in the fed–state (n = 8). (**h**) [^{14}C]–Palmitate oxidation in isolated *extensor digitorum longus* muscle (n = 8–10). (**i**) Skeletal muscle DAG and TAG (n = 6–8). (**j**) The serum activities of markers of liver function, alanine aminotransferase (ALT) as a ratio of aspartate aminotransferase (AST) between WT control and AccDKI (n = 7–8). Data are expressed as means ± SEM, where * $P < 0.05$ and ** $P < 0.01$ compared to WT control, $^{\#}$ $P < 0.05$ compared to basal treatment as determined by Student's t test or an ANOVA and Bonferonni *post hoc* test, respectively. *Mcd* relative gene expression was normalized to *Actb*.

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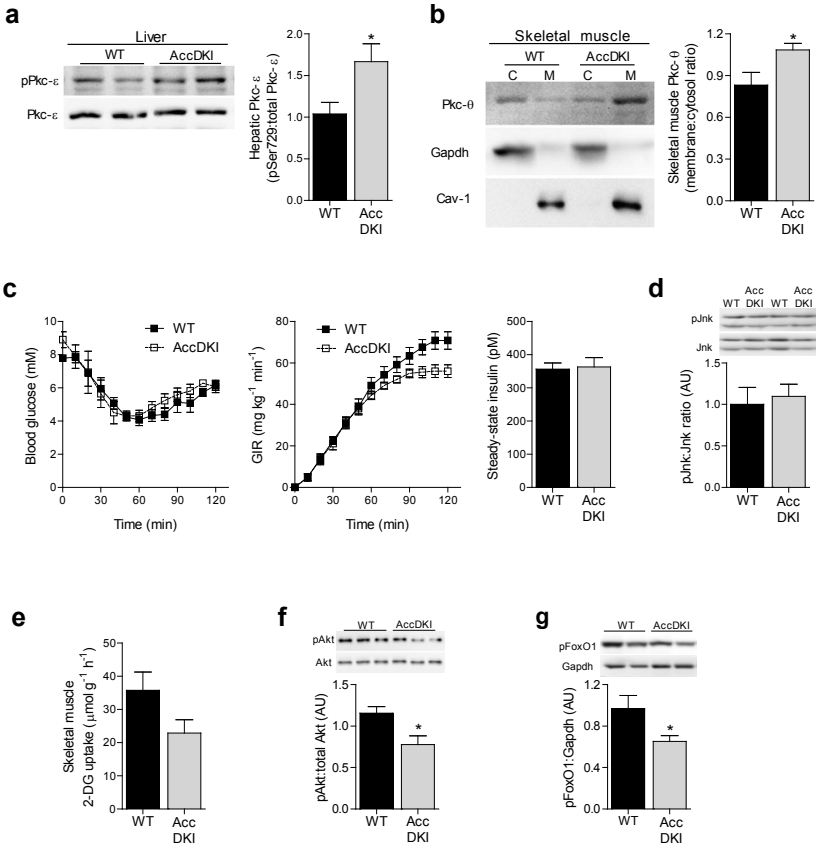
Supplementary Figure 3



Supplementary Fig. 3. Lipid parameters, Pkc activation and insulin sensitivity in chow mice. (**a**) Liver Pkc–ε Ser729 phosphorylation (n = 7). (**b**) Skeletal muscle membrane–associated Pkc–θ (n = 7), shown as the ratio of membrane:cytosolic fraction. (**c**) Blood glucose measurements and glucose infusion rates (GIR) over the time course of the clamp procedure, as well as steady–state insulin levels at the termination of the clamp for chow–fed mice (n = 7–8). (**d**) Representative blot and densitometry showing phosphorylation of liver Jnk (n = 5). (**e**) [^{14}C]–2–deoxyglucose (2–DG) uptake into skeletal muscle during the clamp (n = 7–8), and skeletal muscle (**f**) Akt (Ser473) phosphorylation and (**g**) FoxO1 (Ser253) phosphorylation at the completion of the clamp (n = 7–8). Data are expressed as means ± SEM, where * P < 0.05 compared to WT control as determined by a Student's t test. Duplicate gels were run for quantification of total Akt and Jnk. For Pkc activation, Gapdh and caveolin–1 were used for cytosolic and membrane normalization, respectively, and blots shown are from duplicate gels.

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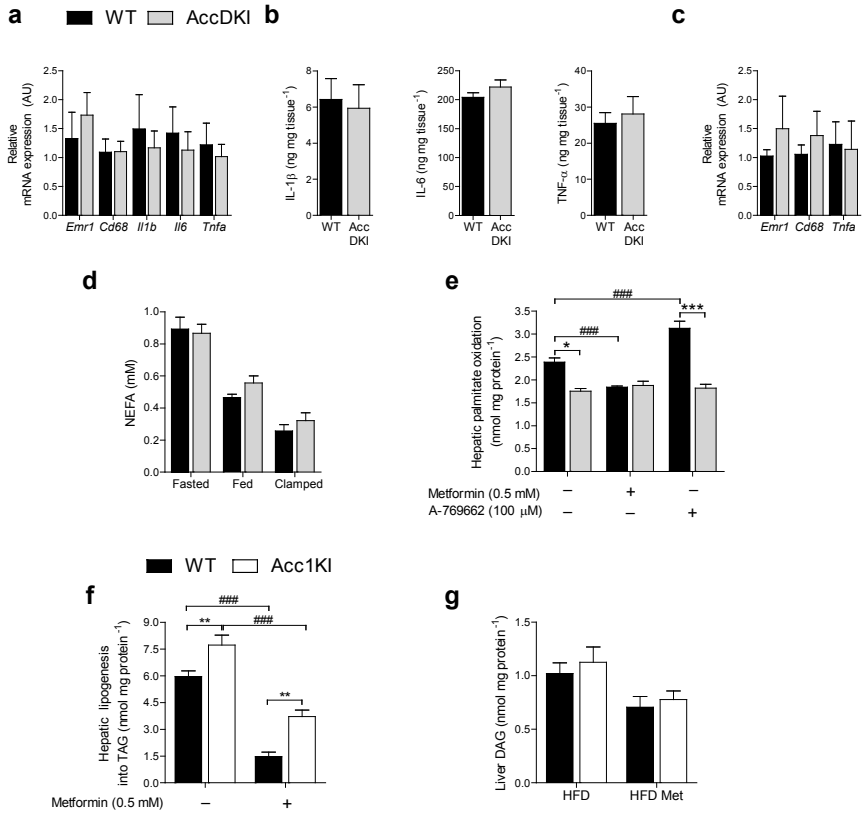
Supplementary Figure 4



Supplementary Fig. 4. AccDKI insulin resistance is not due to inflammation or higher serum fatty acids, and metformin requires Acc1 and Acc2 phosphorylation. (**a**) The relative expression of macrophage–specific transcripts as well as pro–inflammatory cytokines in the liver (n = 7–8). (**b**) Hepatic pro–inflammatory cytokine protein levels IL–1β, IL–6 and TNF–α (n = 8). (**c**) The relative expression of macrophage–specific transcripts as well as pro–inflammatory cytokines in the epididymal white adipose tissue (WAT) (n = 7–8). (**d**) Circulating non–esterified fatty acids (NEFA) in the fasted–, fed– and clamped–state (n = 7–16). (**e**) [^{14}C]–palmitate oxidation from WT and AccDKI hepatocytes (n = 3–4 from at least 2 separate experiments). (**f**) [^{3}H]–acetate incorporation into TAG was measured from WT and Acc1KI hepatocytes (n = 3 from 2 separate experiments). (**g**) Hepatic DAG from HFD–fed WT and Acc1KI mice chronically treated with 50 mg/kg metformin (n = 8–12). Data are expressed as mean ± SEM, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to WT and $^{###}$ $P < 0.001$ compared to vehicle control as determined by ANOVA and Bonferonni *post hoc* test, and relative gene expression was normalized to *Actb*.

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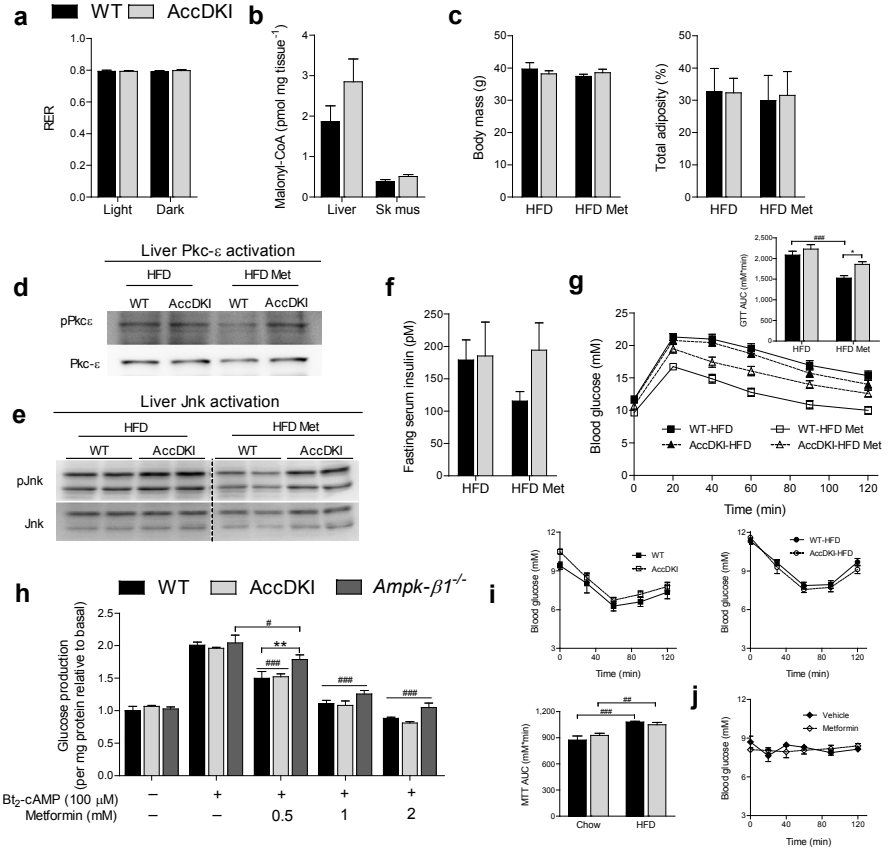
Supplementary Figure 5



Supplementary Fig. 5. Lipid metabolism is normalized in AccDKI HFD mice and the acute effects of metformin to suppress hepatic glucose are independent of Acc phosphorylation. (**a**) Respiratory exchange ratio (RER) from HFD-fed WT and AccDKI mice. (**b**) Liver and skeletal muscle malonyl–CoA from HFD–fed WT and AccDKI mice in the fed–state (n = 8). (**c**) Final body mass and percent adiposity (n = 10). (**d**) Phosphorylated Pkc–ε (Ser729) and (**e**) phosphorylation of Jnk in the liver of HFD and HFD–metformin treated mice (n = 7). (**f**) Fasting serum insulin levels and (**g**) glucose tolerance test (1 g/kg) and area under the curve (n = 10). (**h**) Glucose production in primary hepatocytes treated with or without increasing concentrations of metformin (n = 5 from at least 2 separate experiments). (**i**) Metformin tolerance test (200 mg/kg) in overnight–fasted WT and AccDKI mice fed a regular chow diet (n = 5), or a HFD (n = 7–8), with associated area under the curve. (**j**) Metformin tolerance test (50 mg/kg or saline vehicle) in overnight–fasted WT mice fed a regular chow diet (n = 5). Data are expressed as mean ± SEM, for area under the curve, * P < 0.05 compared to WT and $^{\#\#}$ P < 0.01 and $^{\#\#\#}$ P < 0.001 compared to control diet. For hepatocytes, ** P < 0.01 compared to WT and $^{\#}$ P < 0.05 and $^{\#\#\#}$ P < 0.001 compared to no Bt_2–cAMP control as determined by ANOVA and Bonferonni *post hoc* test. Duplicate gels were run for quantification of total Pkc–ε and Jnk.

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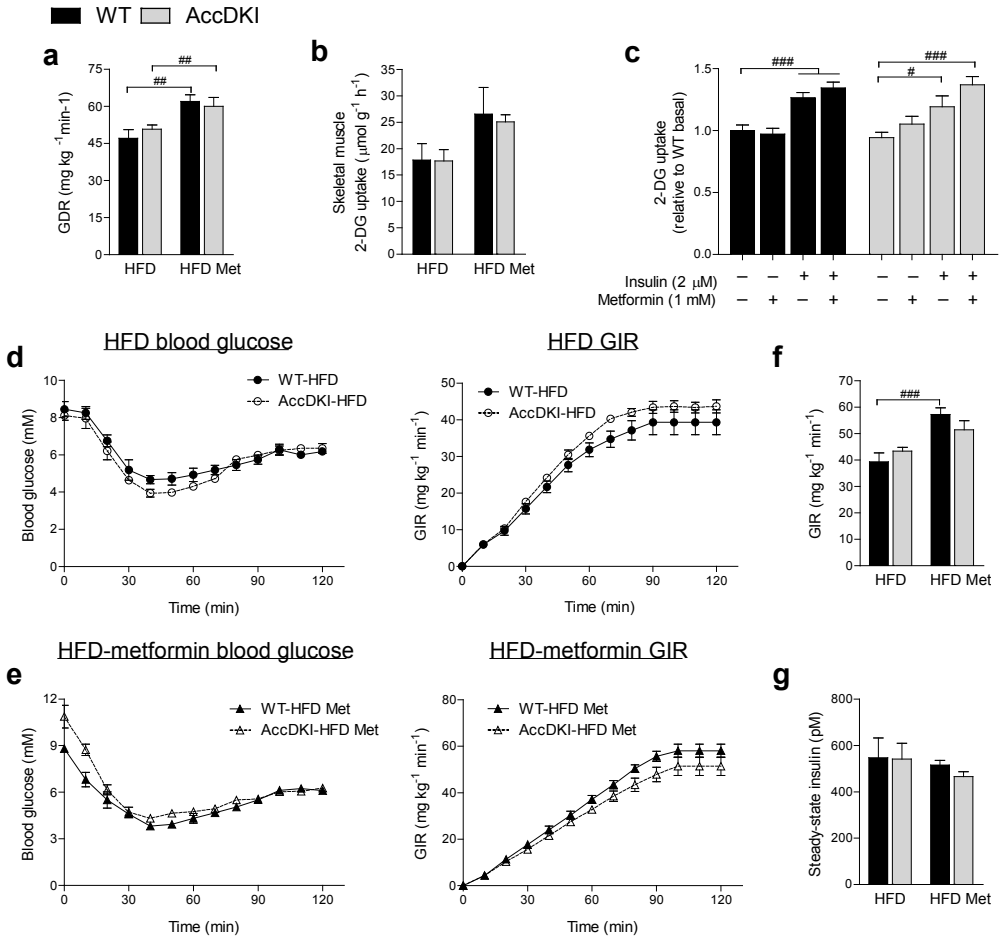
Supplementary Figure 6



Supplementary Fig. 6. Chronic metformin treatment increases skeletal muscle glucose uptake independent of Acc phosphorylation. (**a**) Glucose disposal rate (GDR) for HFD–control and HFD–metformin treated WT and AccDKI mice (n = 7–8). (**b**) [^{14}C]–2–deoxyglucose (2–DG) uptake into skeletal muscle during the clamp (n = 7–8). (**c**) [^{3}H]–2–DG uptake in isolated *ex vivo* stimulated *extensor digitorum longus* muscle from WT or AccDKI mice (n = 5–6). Hyperinsulinemic–euglycemic clamp results for blood glucose measurements and glucose infusion rate (GIR) over the time course of the clamp procedure for (**d**) HFD control, (**e**) HFD–metformin treated WT and AccDKI mice and (**f**) a summary of final rates (n = 7–8). (**g**) Steady–state insulin levels at the termination of the clamp (n = 7–8). Data are expressed as mean ± SEM, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to control treatment as determined by ANOVA and Bonferonni *post hoc* test.

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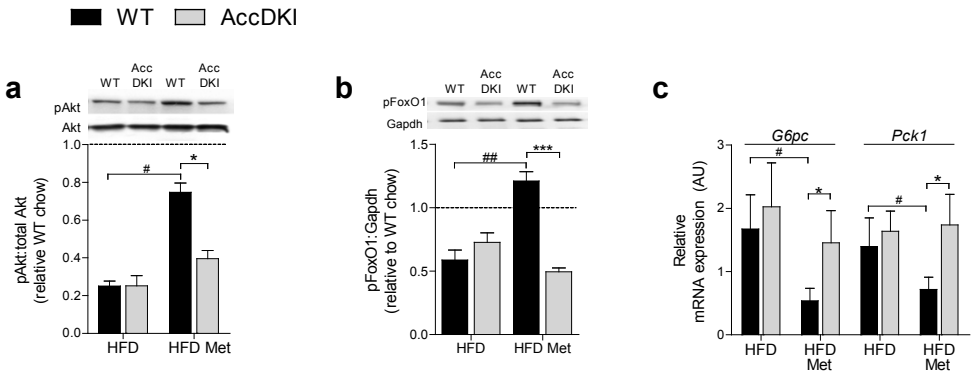
Supplementary Figure 7



Supplementary Fig. 7. Chronic metformin treatment improves hepatic insulin action in WT mice, but not AccDKI. (**a**) Liver Akt (Ser473) phosphorylation, (**b**) liver FoxO1 (Ser253) phosphorylation shown relative to chow WT control (n = 5) and (**c**) gluconeogenic gene expression (*G6pc* and *Pck1*) at the completion of the clamp (n = 8). Data are expressed as means ± SEM, * $P < 0.05$ and *** $P < 0.001$ represent differences between genotype, and $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ are differences between treatment as determined by ANOVA and Bonferonni *post hoc* test. Relative gene expression was normalized to *Actb* and duplicate gels were run for quantification of total Akt and Gapdh.

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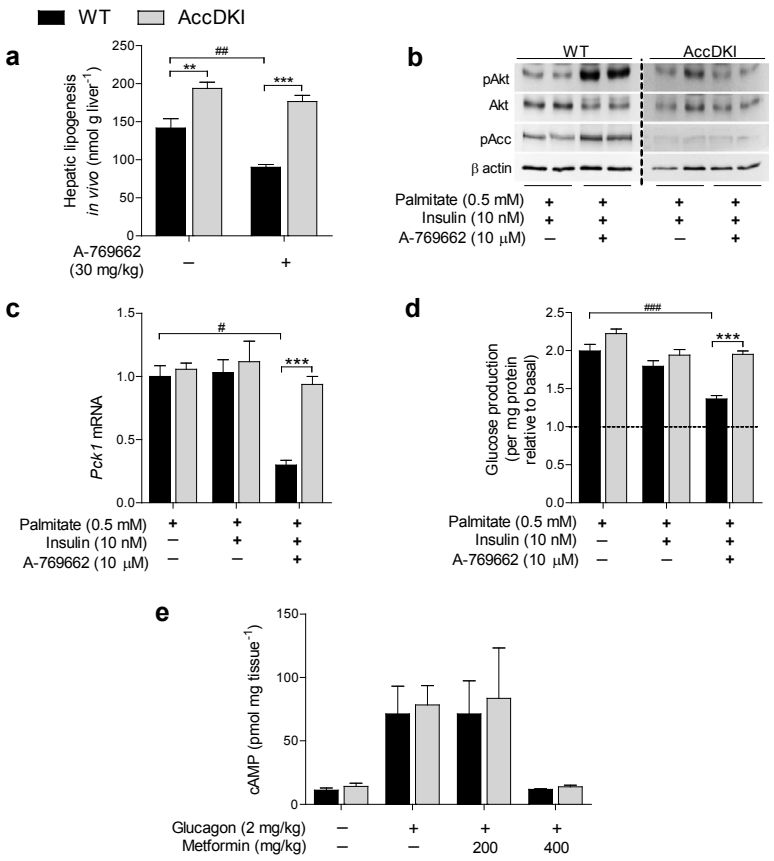
Supplementary Figure 8



Supplementary Fig 8. Specific Ampk activation requires Acc signaling, but metformin–induced suppression of glucagon-stimulated cAMP does not. (**a**) *In vivo* incorporation of [^{3}H]–acetate into total liver lipid (*de novo* lipogenesis) in WT and AccDKI mice treated with vehicle (5% DMSO in PBS) or A–769662 (30 mg/kg) (n = 4 for vehicle and n = 5 for A–769662). Primary hepatocytes were treated ± A–769662 (10 μM) in the presence of palmitate (0.5 mM) for 18 h and insulin (10 nM) stimulation of (**b**) Akt (Ser473) phosphorylation or suppression of (**c**) *Pck1* expression determined (shown relative to WT, palmitate control) (n = 2–4). (**d**) Insulin suppression of hepatic glucose production, following chronic (18 h) exposure to palmitate (0.5 mM) ± A–769662 (10 μM) (n = 3–4, from at least 2 separate experiments). Hatched line represents no Bt_2–cAMP. (**e**) Hepatic glucagon–stimulated cAMP in WT and AccDKI mice administered vehicle (saline), 200 or 400 mg/kg metformin in the fed–state were (n = 3–4). Data are expressed as means ± SEM, ** $P < 0.01$ and *** $P < 0.001$ represent differences between genotype, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ are differences between treatment, as determined by ANOVA and Bonferonni *post hoc* test. Duplicate gels were run for total Akt and β actin, where all samples from both genotypes were run on the same gel, but WT and DKI samples were imaged separately.

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CHAPTER THREE

Exercise training improves liver insulin sensitivity independently of liver lipid content, inflammation and AMPK-Acetyl-CoA Carboxylase signaling

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This manuscript examines the effects of exercise training on insulin sensitivity and fatty acid metabolism in WT and AccDKI obese mice. Insulin sensitivity and NAFLD are two closely connected complications of obesity. NAFLD has also been associated with the transfer of fatty acids and fatty acid derived metabolites from visceral adipose tissue to the liver via the hepatic portal vein in the 'portal theory'. Findings from prior exercise studies examining NAFLD and adipose tissue inflammation individually have been complicated by weight loss making it difficult to delineate mechanisms by which exercise improves insulin sensitivity and NAFLD. We, therefore, developed a relatively low volume high-intensity interval exercise training program to minimize caloric expenditure and prevent weight loss in both WT and AccDKI mice fed a high-fat diet. We show that exercise training increases food intake, basal activity levels, carbohydrate oxidation and adipose tissue and liver insulin sensitivity without reducing adiposity or liver lipid content. These data indicate that exercise training leads to insulin sensitization of the liver independently of reducing adiposity or markers of NAFLD.

KM, SRS, MF and GRS conception and design of research; KM, SS, MCS, and MDF performed experiments; KM and GRS analyzed data; KM and GRS interpreted results of experiments; KM and GRS prepared figures; KM and GRS drafted manuscript; KM, BEK, MDF and GRS edited and revised manuscript; KM, SRS, BEK, MCS, MDF and GRS approved final version of manuscript. KM and GRS are responsible for the integrity of the work as a whole.

Note: Figure 4C,D,E,F,G,H were competed by SRS.

High intensity interval training improves liver and adipose tissue insulin sensitivity independently of weight loss, liver lipid content or adipose tissue inflammation

Short running title: HIIT improves liver and adipose insulin sensitivity

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Abstract

Objective: Endurance exercise training reduces insulin resistance, adipose tissue inflammation and non-alcoholic fatty liver disease (NAFLD), an effect often associated with modest weight loss. Recent studies have indicated that high-intensity interval training (HIIT) lowers blood glucose in individuals with type 2 diabetes independently of weight loss; however, the organs affected and mechanisms mediating the glucose lowering effects are not known. Intense exercise increases phosphorylation and inhibition of acetyl-CoA carboxylase (ACC) by AMP-activated protein kinase (AMPK) in muscle, adipose tissue and liver. AMPK and ACC are key enzymes regulating fatty acid metabolism, liver fat content, adipose tissue inflammation and insulin sensitivity but the importance of this pathway in regulating insulin sensitivity with HIIT is unknown.

Methods: In the current study, the effects of 6 weeks of HIIT were examined using obese mice with serine-alanine knock-in mutations on the AMPK phosphorylation sites of ACC1 and ACC2 (AccDKI) or wild-type (WT) controls.

Results: HIIT lowered blood glucose and increased exercise capacity, food intake, basal activity levels, carbohydrate oxidation and liver and adipose tissue insulin sensitivity in HFD-fed WT and AccDKI mice. These changes occurred independently of weight loss or reductions in adiposity, inflammation or liver lipid content.

Conclusions: These data indicate that HIIT lowers blood glucose levels by improving adipose and liver insulin sensitivity independently of changes in adiposity, inflammation, liver lipid content or AMPK phosphorylation of ACC.

Keywords HIIT; exercise; obesity-induced insulin resistance; type 2 diabetes; NAFLD; AMPK.

Abbreviations

ACC	Acetyl-CoA carboxylase
AccDKI	Serine-alanine knock-in mutations of ACC1 Ser79 and ACC2 Ser212
ALT	Alanine transaminase
AMPK	AMP-activated protein kinase
AST	Aspartate transaminase
AUC	Area under the curve
CPT-1	Carnitine palmitoyl transportase-1
CT	Computed tomography
DAG	Diacylglycerol
GDR	Glucose disposal rate
GIR	Glucose infusion rate
HFD	High-fat diet (45% kcal fat)
HGP	Hepatic glucose production
HIIT	High-intensity interval training
ITT	Insulin tolerance test
Nefa	Non-esterified fatty acids
RER	Respiratory exchange ratio
TAG	Triacylglycerol
WT	Wildtype

1. Introduction

Endurance exercise training improves insulin sensitivity and delays the onset of type 2 diabetes through mechanisms which are not fully understood [1–3]. Despite the importance of endurance exercise training, less than 20% of individuals complete the recommended 150 minutes of endurance exercise per week, frequently citing a lack of time as a major deterrent [4]. Over the last decade, several studies in humans have found that high-intensity interval training (HIIT), an exercise training program involving brief bouts of intense exercise (90-100% of VO_2 max) followed by periods of recovery, can elicit similar metabolic adaptations to classical endurance exercise training but with a much shorter time commitment. Importantly, recent studies have established that HIIT can lower blood glucose and markers of insulin resistance independently of alterations in adiposity/body mass in individuals with insulin resistance and type 2 diabetes [5–9]. Despite these beneficial metabolic effects, the tissues involved and mechanisms underlying the glucose lowering effects of HIIT have not yet been defined.

Insulin resistance is associated with the development of low grade inflammation caused by an increased accumulation of pro-inflammatory macrophages into adipose tissue and ectopic accumulation of lipid in the liver (also known as non-alcoholic fatty liver disease (NAFLD)) [10–15]. Endurance exercise training can reduce liver lipid content [16–23] and adipose tissue inflammation [10,11,24–27]; however, a caveat of these studies is that they are

often accompanied by significant weight loss/reductions in adiposity [18–25], thus making it difficult to conclude whether improvements were attributable to the exercise training *per se* or weight loss. HIIT improves insulin sensitivity without weight loss, but whether this involves reductions in liver lipid content or adipose tissue inflammation has not been examined.

One mechanism by which HIIT may improve insulin sensitivity involves the activation of AMP-activated protein kinase (AMPK) which occurs in skeletal muscle [28–31], liver [30,32] and adipose tissue [30,33,34] during intense exercise. AMPK is vital for suppressing inflammation in adipose tissue macrophages [35–38], an effect associated with increases in macrophage fatty acid oxidation and reductions in macrophage lipid content. Similarly, the activation of AMPK in hepatocytes also increases fatty acid oxidation, while reducing fatty acid synthesis and liver lipid content [35,39]. The effects of AMPK on fatty acid metabolism are mediated through the phosphorylation and inhibition of acetyl-CoA carboxylase (ACC1) at Ser79 and ACC2 at Ser221 (Ser212 in mice) which inhibits the production of malonyl-CoA, a metabolic intermediate that provides acetyl groups that are incorporated into fatty acids during their synthesis and is also an allosteric inhibitor of carnitine palmitoyltransferase 1 (CPT-1) (for review see [40]). The mutation of AMPK phosphorylation sites on ACC1 (Ser79Ala) and ACC2 (Ser212Ala) (AccDKI mice) results in constitutively active ACC isozymes resulting in fatty and fibrotic liver and impaired insulin sensitivity when mice are fed a control chow diet [39]. Although feeding mice a

high-fat diet (HFD) reduces the differences in metabolic profile between WT and AccDKI mice, metformin was shown to improve insulin sensitivity through ACC phosphorylation and subsequent reductions in *de novo* lipogenesis and liver lipid content [39]. Whether or not exercise training also regulates inflammation, liver lipid content and insulin sensitivity via an AMPK-ACC signaling pathway is currently unknown.

The primary aim of this study was to assess the mechanisms by which HIIT improves insulin sensitivity in obese mice. We hypothesized that this would involve improvements in adipose tissue and liver insulin sensitivity, effects that would be mediated through the phosphorylation and inhibition of ACC and subsequent reductions in liver lipid content and adipose tissue inflammation. We found that HIIT improved liver and adipose tissue insulin sensitivity but that these effects were independent of liver lipid content, adipose tissue inflammation and ACC phosphorylation.

2. Materials and Methods

2.1 Mouse experiments. Male AccDKI (serine-alanine knock-in mutations of ACC1 Ser79 and ACC2 Ser212) mice generated on a C57Bl/6 background and wild-type (WT) littermates were first fed ad libitum with high-fat diet (HFD) (45 kcal% fat, D12451, Research Diets; New Brunswick, NJ). Mice were maintained on a 12 hour light/dark cycle and fed a HFD starting at 6-8 weeks of age for 12 weeks. After the first 6 weeks of HFD, mice were either exercise trained or

remained sedentary for the final 6 weeks. All experiments were approved by the McMaster University (Hamilton, Canada) Animal Ethics Committee.

2.2 Exercise capacity and HIIT. Mice assigned to the HIIT exercise training (HFD+Ex) group were acclimatized to the treadmill over 3 days, running at 10-15 m/min for 15 minutes. To assess improvements in exercise performance with training, an exercise capacity test was performed before training and after 5 weeks of training. Mice began treadmill running at 8 m/min and treadmill speed was increased by 1 m/min every 2 minutes until exhaustion. Exhaustion was defined as the point at which instead of running on the treadmill, mice remained on the shockers that serve to encourage running for more than 10 seconds. At exhaustion, time and speed were recorded. Distance traversed was calculated by adding the distance covered during each 2 minute interval at the different workloads/treadmill speed. The experimenter was blinded to the mouse genotypes.

HIIT involved treadmill running 3 days per week for the final 6 weeks of HFD. Exercise training entailed 2 min of running at 100% of maximal running speed from the initial exercise capacity test followed by 2 min of rest for a total 60 min. This meant that HFD+Ex mice ran on the treadmill at 15 m/min for 2 minutes followed by 2 minutes of rest for a total of 60 min during the first week. The speed of running was increased by 1 m/min every week with a final speed of 22 m/min obtained during the final week of training. During the period that mice were trained, the sedentary group remained in their cages and ate HFD *ad libitum*.

2.3 Metabolic parameters. The Oxymax Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH) uses indirect calorimetry to measure metabolic gas exchange, ambient activity, and food intake as described previously [35]. Measurements began ~24 hours after the latest exercise session to avoid any post-exercise effects. Mice were acclimatized to the cages for 12 hours prior to measurements. Basal metabolic rate was the VO_2 when mice were inactive (as determined by ≤100 beam breaks per minute) as we have described previously [41].

2.4 Metabolic studies. To detect acute phosphorylation of AMPK and ACC in the liver of obese mice fed a HFD for 6 weeks, liver was collected and snap frozen immediately after an acute bout of HIIT exercise. For our chronic training, body mass of mice was monitored and recorded weekly. Computed tomography (CT) was used to assess the effects of exercise training on whole body adiposity and analyzed with the Amira Visage Imaging Software Program, as described previously [42]. Blood (~100 μL) was collected by facial bleed in the fed and 12 hour fasted state (with fasting beginning in the evening), after 5 and 6 weeks of exercise training, respectively. Blood glucose concentrations were recorded by hand-held glucometer. Serum analysis of 12 hour fasting insulin (Millipore) was performed according to manufacturer instructions. Serum analysis of alanine transaminase (ALT) and aspartate transaminase (AST) after 12 hours of fasting was conducted as per manufacturer instructions (Biooscientific). Serum adipokines (interleukin-6 (IL-6), leptin, tissue plasminogen activator inhibitor-1

(tPAI-1), resistin, tumor necrosis alpha (TNF-α), and monocyte chemoattractant protein-1/CCL2 (MCP-1)) were measured using the Mouse Serum Adipokine kit from Millipore (St. Charles, MO), according to manufacturer's instructions. In addition, intraperitoneal (ip) glucose (D-glucose (1 g/kg)) and insulin (human insulin (1 U/kg, NovoRapid)) tolerance tests were performed after a 6 hour fast after 5 and 6 weeks of exercise training, respectively. Blood glucose was measured by glucometer from a small nick of the tail vein during a 2 hour span after ip injection. After 12 weeks of the study with 6 weeks of exercise training and a cannulation of the jugular vein surgery (with <10% loss in body weight change), hyperinsulinemic-euglycemic clamps were performed as previously described [35,39]. After a 5 hour fast and 1 hour of basal D- [3-^{3}H]-glucose (7.5 μCi/h, 0.12 ml/h in 0.9% saline) infusion, a constant infusion of insulin (10 mU/kg/min insulin in 0.9% saline) (Novorapid), containing D- [3-^{3}H]-glucose (7.5 μCi/h, 0.12 ml/h in 0.9% saline) was begun. This was followed by an infusion of 50% dextrose that was slowly increased until euglycemia - blood glucose between 5.8-7.0 mM for at least 30 minutes - was reached [35]. Steele's equation for steady state conditions was used to determine rates of hepatic glucose output and glucose disposal in the basal and clamped state [43]. Finally, clamped blood was collected and serum was used to measure non-esterified fatty acid (NEFA) concentration, as per manufacturer instructions (Wako). 2-deoxyglucose (DG) uptake into epididymal white adipose tissue (eWAT) and mixed gastrocnemius muscle was measured following an intravenous injection of 2-

[^{14}C]-DG (10 μCi) at the conclusion of the clamp as we have described previously [39]. Briefly, blood samples were taken at 10, 20, and 30 minutes. Mice were euthanized with an intravenous injection of ketamine-xylazine and tissues were collected, snap frozen in liquid nitrogen, and stored at minus 80°C for later analysis [42]. eWAT and gastrocnemius muscle lysates were prepared and glucose uptake was quantified via scintillation counting in which the unphosphorylated 2-[^{14}C] DG fraction was subtracted from the total fraction to give the quantity of 2-[^{14}C] DG-P. 2-[^{14}C] DG-P was then expressed relative to blood glucose and 2-[^{14}C] DG infusion in the blood.

2.5 Analytical Techniques. Liver tissue was powdered on dry ice and homogenized in cell lysis buffer using a Precellys 24 Homogenizer (Bertin Technologies; Paris, France). To quantify liver triacylglycerol (TAG) content, lipids were extracted with chloroform and methanol [44], were saponified and glycerol content measured using Glycerol Reagent (Sigma). Diacylglycerols (DAG) and ceramides were quantified using the DAG kinase assay, as previously described [45]. Briefly, lipids were extracted from freeze-dried liver and gastrocnemius muscle tissue sample that was incubated in chloroform:methanol:0.2% SDS (1:2:0.8) in PBS. Cardiolipin/octylglucoside was used to reconstitute the lipids and [γ-^{32}P] ATP reaction mixture was added. The reaction was stopped with chloroform:methanol (2:1) and samples were separated by thin layer chromatography as described previously [42]. To measure glycogen

content, liver tissue was incubated in 6N HCl at 80°C and then neutralized with 6N NaOH. Glucose content was then measured using a Glucose Assay (Sigma).

2.6 Adipose tissue macrophage collection. Macrophages display the unique dendritic-cell specific marker CD11b and CD11c in obese mice and humans and, for simplicity, they are often grouped either as classically or alternatively activated [10,11]. Adipose tissue macrophages were isolated as previously described [35]. Briefly, adipose tissue was minced and digested with Type II Collagenase (Sigma). The resulting cell suspension was filtered (100μm cell strainer) and centrifuged (500g for 5 minutes). Pellet was re-suspended in recommended medium (2% FBS, 1mM EDTA) and labeled with CD11b+ PE Labeling Reagent, PE Selection Cocktail, and Magnetic Nanoparticles (StemCell Technologies; Vancouver, BC). Macrophages were separated using the EasySep® kit magnet. Macrophages were resuspended in 1mL TRIzol® Reagent (Invitrogen; Carlsbad, CA) and stored at -80°C.

2.7 Histology and Immunohistochemistry. A small piece of liver and adipose tissue were dissected and formalin-fixed in 10% buffered formalin. Paraffin-embedding and slicing of 5 μm thick sections were completed by trained technicians in the histopathological lab in the Department of Medicine at McMaster University. Sections of liver and adipose tissue were stained with hematoxylin and eosin (H&E) to assess the overall degree of steatosis compared between groups. In addition, a section of liver was made for trichrome staining. Collagen content (area fibrosis) was quantified using ImageJ software. Sections of

adipose tissue were also prepared for immunohistochemistry (IHC) to assess macrophage infiltration in the adipose tissue. Paraffin-embedded adipose tissue was dewaxed and rehydrated to perform antigen retrieval by boiling (15 minutes) the slides in 10mM sodium citrate (pH 6.5). Endogenous peroxidase was quenched (1% fetal calf serum and 3% hydrogen peroxide in PBS) and tissue samples were then blocked (5% normal rabbit serum) for 40 minutes, incubated with Avidin D (Biotin/Avidin Blocking Kit, Vector Laboratories; Burlinghame, CA) for 15 minutes, and incubated with Biotin (Biotin/Avidin Blocking Kit, Vector Laboratories) for 15 minutes. Sections were then incubated with primary antibody rat anti-mouse F4/80 (1:100) (AbD Serotec, Oxford, UK) for 2 hours, a biotin-conjugated secondary antibody (1:50) (Vector Laboratories) for 1 hour, and Vectastain ABC solution (Vector Laboratories) for 30 minutes. The sections were developed using the DAB Substrate Kit (Vector Laboratories) sections and slides were counterstained with hematoxylin.

2.8 Real-time quantitative PCR. RNA from liver, adipose tissue, and macrophages were extracted using TRIzol reagent and cDNA was made using Superscript III Reverse Transcriptase (Invitrogen), according to manufacturer instructions. TaqMan® Gene Expression Assays (Applied Biosystems; Foster City, CA) were used for real-time quantification with Rotor-Gene 6000 (Corbett Research; Mortlake, Australia) of (1) liver glucose 6-phosphatase (*G6pc*, Mm00839363_m1), phosphoenolpyruvate carboxykinase (*Pck1*, Mm01247058_m1), interleukin 1 beta (*Il1β*, Mm00434228_m1), tumor necrosis

factor alpha (*Tnfa*, Mm00443258_m1), F4/80 (*Emr1*, Mm00802529_m1), interleukin 10 (*Il10*, Mm00439616_m1), macrophage arginase (*Arg*, Mm01190441_g1), inducible nitric oxide synthase (*Nos2*, Mm01309902_m1), *Cd68* (Mm00839636_g1), and *(Kc*, Mm00433859_m1) genes; and (2) adipose tissue interleukin-6 (*Il6*, Mm00446190_m1), *Cd68* (Mm00839636_g1), and F4/80 (*Emr1*, Mm00802529_m1), uncoupling protein 1 (*Ucp1*, Mm01244861_m1), cell death activator (*Cidea*, Mm00432554_m1), PR domain containing 16 (*Prdm16*, Mm00712556_m1), pyruvate dehydrogenase kinase isoform 4 (*Pdk4*, Mm01166879_m1), peroxisome proliferator-activated receptor gamma coactivator-1-alpha (*Ppargc1a*, Mm00447183_m1), glucose transporter 4 (*Glut4*, Mm00436615_m1); adipose tissue macrophage arginase (*Arg*, Mm01190441_g1), inducible nitric oxide synthase (*Nos2*, Mm01309902_m1), interleukin 1 beta (*Il1β*); and adipose tissue and macrophage, chemokine ligand 1 (*Kc*, Mm00433859_m1), and tumor necrosis factor alpha (*Tnfa*, Mm00443258_m1). Gene expression was measured using the ddCT method[46], in which the expression of a gene of interest is compared to that of the housekeeping gene (*CycA* - Mm00839493_m1, *Tbp* - Mm00446973_m1 and *Rplp0* - Mm01974474_gH) and expressed relative to HFD WT.

2.9 Western blotting. Protein extracts were separated by SDS-PAGE, and immunoblotting was performed after transfer to nitrocellulose membranes. Membranes were blocked for 1 hour at room temperature with 5% BSA in 1x TBST (Tris buffered saline-Tween-20, 25 mM Tris·HCl (pH 7.5), 1 mM NaCl,

and 0.1% Tween-20) and then incubated overnight at 4°C with primary antibodies (from Cell Signaling, unless indicated) phosphorylated (p)-acetyl-CoA carboxylase (ACC) Ser79/221 (1:1000), ACC total (1:1000), pAMPK Thr172 (1:1000), AMPK total (1:1000), pAkt Ser473 (1:1000), pAkt Thr308 (1:1000), Akt (1:1000), pAS160 (Akt substrate of 160 kDa) Thr642, (1:1000), OXPHOS (MitoSciences, 1:5000), anti-PGC-1 (Millipore, 1:1000) in liver and/or quadriceps muscle. After overnight incubation, membranes were washed (3 x 5 min) in TBST, incubated at room temperature for 1-hr with corresponding secondary antibody, washed (3 x 5 min) with TBST and developed with Clarity^{TM} Western ECL Substrate (Biorad). Protein expression was shown relative to GAPDH (Cell Signaling, 1:5000). Densitometry was performed using ImageJ software.

2.10 Statistical analysis. Results are expressed as mean ± standard error of the mean (SEM) and analyzed using a two-way ANOVA with Bonferroni post-hoc tests for group comparisons (unless otherwise denoted), using GraphPad Prism software. Repeated measures ANOVA was used to measure body mass, GTT, ITT, and GIR. Significance was accepted at $p \leq 0.05$.

3. Results

3.1 Exercise capacity is increased with HIIT independent of body weight and adiposity. To first establish that the HIIT activated AMPK, we collected liver from WT mice following a single training session and found increased AMPK (Supplementary Figure 1A) and ACC (Supplementary Figure 1B)

phosphorylation. All subsequent data were collected in AccDKI or WT littermates fed a HFD that were either sedentary or completed the HIIT protocol 3 times per week for 6 weeks. Consistent with our previous report [39], we found that WT and AccDKI mice had comparable weight gain and adiposity when fed HFD (Figure 1A,B). Exercise training did not alter these variables in either genotype (Figure 1A,B). There was no difference in liver, eWAT or heart tissue weights between any group with or without HIIT (Table 1). Furthermore, HFD-fed WT and AccDKI mice did not show differences in exercise capacity and both improved time to exhaustion, speed at exhaustion, and distance traversed to an equivalent degree following HIIT (Figure 1C,D,E). This improvement in exercise capacity is comparable to what other studies have observed using higher volume endurance exercise training programs [18,47].

3.2 HIIT improves metabolic flexibility independently of AMPK phosphorylation of ACC. Energy intake and expenditure, activity levels and substrate utilization were examined using metabolic cages. We found that HIIT increased food intake and ambient activity in both WT and AccDKI mice (Figure 1F,G). Consistent with increased ambient activity, exercise trained mice had increased energy expenditure (Figure 1H) in line with elevations in VO_2 and VCO_2 (Figure 1I). This increase in VO_2 was not due to alterations in basal metabolic rate, which was comparable between sedentary and exercise trained mice irrespective of genotype (Figure 1J), suggesting that increases in VO_2 were

the result of an increase in activity levels. There were no differences in respiratory exchange ratio (RER) (Figure 1K), carbohydrate oxidation (Figure 1L), or lipid oxidation (Figure 1M) between sedentary HFD-fed WT and AccDKI mice. However, HIIT increased RER and calculated rates of carbohydrate oxidation while suppressing lipid oxidation in both WT and AccDKI mice (Figure 1K,L,M), findings suggestive of improved whole-body insulin sensitivity following exercise training [48,49].

3.3 HIIT improves insulin sensitivity independently of AMPK phosphorylation of ACC. All of the assessments of glucose homeostasis were completed 48-72 hours after treadmill running to minimize the acute insulin sensitizing actions of exercise. Consistent with our previous findings [39], sedentary HFD-fed WT and AccDKI mice had comparable fed blood glucose (Figure 2A), fasting blood glucose and serum insulin levels (Figure 2B,C). Insulin tolerance test (ITT), glucose tolerance test (GTT), and corresponding area under the curves (AUCs) were also comparable between WT and AccDKI mice (Figure 2D-I). While no chow group was examined in this study, these measures of glucose and insulin were all much higher than we have previously reported in chow fed WT and AccDKI mice [39] or other mice fed a chow diet in our laboratory [50], indicating the development of insulin resistance following the 12 wks of HFD. Importantly, HIIT reduced fed (Figure 2A) and 12 hour fasted (Figure 2B) blood glucose levels and tended to improve fasting serum insulin

levels (Figure 2C). Insulin tolerance tests indicated improvements in whole-body insulin sensitivity by ~20% in both WT and AccDKI mice following HIIT (Figure 2D,E,F); however, surprisingly, glucose tolerance was not significantly different (Figure 2G,H,I).

3.4 HIIT increases adipose tissue glucose uptake without altering inflammation, macrophage infiltration or browning markers. To examine the mechanisms mediating improvements in insulin sensitivity, we conducted hyperinsulinemic-euglycemic clamps. An important caveat of this experiment is that based on the time to catheterize the mice and allow recovery, the clamp was completed 5 days after the last exercise bout and, as such, significant detraining effects may have occurred, thus minimizing differences between the sedentary and HIIT intervention groups. In the clamped state, glucose infusion rates (GIR) were comparable between sedentary WT and AccDKI mice but, importantly, increased with HIIT indicating improvements in whole-body insulin sensitivity (Figure 3A and Supplementary Figure 1C-F). Surprisingly, glucose disposal rates (GDR) were not different between WT and AccDKI mice and only tended to increase in WT mice that were exercise trained (Interaction p=0.12) (Figure 3B). Consistent with a similar GDR, 2-DG uptake into mixed gastrocnemius muscle was comparable between genotypes (Figure 3C). Skeletal muscle insulin resistance has been associated with long chain acyl-CoA, DAG and ceramide accumulation [51,52]. Although there was no difference either in TAG or

ceramide accumulation in skeletal muscle, HIIT training resulted in reductions in ceramide content in both WT and AccDKI mice (Figure 3D,E). To further assess muscle insulin sensitivity, the phosphorylation of Akt Ser473, Akt Thr308, and AS160/TBC1D4 Thr642 was determined in clamped quadriceps muscle (Figure 3F). HIIT training modestly increased Akt Ser473 and Thr308 phosphorylation,; however, surprisingly, the phosphorylation of its downstream substrate AS160/TBC1D4 phosphorylation was not enhanced (Figure 3F). This suggests that while improving proximal components of the insulin signaling cascade, HIIT training was unable to fully rescue HFD-induced suppression of AS160/TBC1D4 phosphorylation, a finding consistent with the similar 2-DG uptake and GDR in the hyperinsulinemic-euglycemic clamp between sedentary and HIIT trained mice. Future studies investigating the mechanisms contributing to this differential response are warranted.

As muscle mitochondrial content is a robust measure of the exercise training response, we measured protein expression of complexes of the electron transport chain (OXPHOS) in quadriceps muscle. We found that AccDKI mice tended to have higher OXPHOS expression (Figure 3G); a finding consistent with their modest fiber type shift [53]. However, HIIT training only had a tendency to increase OXPHOS protein expression (Figure 3G). Consistent with similar OXPHOS expression with exercise training, PGC1-α protein expression was also unchanged with HIIT training (Figure 3H). Given the robust increase in exercise capacity (which was measured within 72 hours of the last exercise bout), these

data suggest that detraining may have occurred between the last bout of exercise and the day of sacrifice at the completion of the clamps. This rapid detraining effects is consistent with other studies, which have also shown rapid reductions in mitochondrial markers in skeletal muscle following the cessation of exercise training [54–57].

In contrast to the muscle, HIIT dramatically improved eWAT insulin sensitivity, as indicated by increased 2-DG uptake (Figure 4A) and lower circulating NEFA levels (Figure 4B) during the clamp. eWAT size did not differ between WT and AccDKI mice with or without exercise training (Figure 4C). We hypothesized that exercise training improved adipose tissue insulin sensitivity by reducing the number and inflammatory status of adipose macrophages. However, we found that exercise training did not alter the mRNA expression of the macrophage markers *CD68* and *F4/80 (Emr1)* (Figure 4D) or the protein expression of F4/80+ cells in eWAT (Figure 4E), suggesting that HIIT did not influence macrophage accumulation in adipose tissue. There were also no differences in the eWAT mRNA expression of *Il6, Tnfa* or the chemoattractant *Kc* with HIIT (Figure 4F). Serum adipokine levels of IL-6, tPAI01, and resistin also did not differ between mice with or without training (Table 2) with the exception of leptin (Interaction p=0.01), which was lower in WT mice following HIIT (Table 2). These data indicate that HIIT improves adipose tissue insulin sensitivity independently of ACC phosphorylation and without altering macrophage infiltration or markers of adipose tissue inflammation.

Since adipose tissue macrophages are the primary source of inflammation in eWAT, we next isolated this cell type (using a CD11b positive selection kit) and examined markers of polarization by assessing arginase and iNOS. Arginase is an anti-inflammatory marker expressed with alternatively activated (M2) macrophages, and iNOS is a pro-inflammatory marker expressed with classically activated (M1) macrophages. Exercise training did not change *Arg1* or *iNos* expression in adipose tissue macrophages (Figure 4G) nor did it affect the expression of *Tnfa* and *Il1b* (Figure 4H). There was a strong tendency for the chemoattractant *Kc* to be reduced with exercise training (p=0.07) (Figure 4H). Collectively, these data suggest that HIIT does not alter adipose tissue macrophage inflammation.

Although eWAT is resistant to stimuli that induce "browning" it was possible that increased 2-DG uptake into this fat pad following exercise training could potentially be mediated by an increased population of metabolically active beige/brite adipocytes [58,59]. However, we were not able to detect *Ucp1* (data not shown) and there was no difference in the mRNA expression of *Cidea*, *Prdm16*, *Pdk4* and *Ppargc1a* irrespective of genotype or training status (Figure 4I). HIIT training also did not result in changes in *Glut4* expression (Figure 4I).

3.5 HIIT improves liver insulin sensitivity independently of alterations in liver fat content or inflammation. In sedentary mice, hepatic glucose production (HGP) and percent suppression did not differ between WT and AccDKI mice,

consistent with our previous findings [39]. Importantly, exercise training resulted in reduced HGP and greater percent suppression of HGP by insulin in both WT and AccDKI mice (Figure 5A,B). Consistent with greater reductions in HGP, the mRNA expression of the gluconeogenic enzyme *G6pase* was reduced and there was a strong tendency (p=0.06) for reduced *Pepck* expression in the livers of clamped WT and AccDKI mice following exercise training (Figure 5C).

We hypothesized that improvements in liver insulin sensitivity would be accompanied by reductions in liver lipid content but found that surprisingly TAG, DAG, and ceramide content in the liver were comparable (Figure 5D,E). Histological examination of the livers supported similar amounts of lipid deposition and there were no changes in liver fibrosis with HIIT or between genotypes (Figure 5F). No differences were observed in liver glycogen content (Figure 5G). Consistent with similar degrees of liver lipid and fibrosis, HIIT did not reduce the expression of a number of markers of liver inflammatory status (*Il1b*, *Il10*, *iNos*, and *Arg* - Table 3) and, surprisingly, increased the expression of *Tnfa* and *F4/80 (Emr1)* (Table 3). However, the monocyte/macrophage markers *Cd68* and *Kc* were lower in both WT and AccDKI mice that were exercise trained (Table 3). Surprisingly, despite similar lipid content and fibrosis, there was a reduction in serum ALT and AST levels with HIIT in both WT and AccDKI mice (Figure 5H) and a reduction in the ALT/AST ratio (Figure 5I).

4. Discussion

Consistent with studies in humans, we found that HIIT led to a substantial improvement in exercise capacity without altering body mass or adiposity and that this was accompanied by significant reductions in fasting and fed blood glucose levels. HIIT improved adipose tissue insulin sensitivity independently of adipocyte cell size, macrophage accumulation in adipose tissue, adipose tissue inflammation or markers of adipose tissue browning. HIIT also improved liver insulin sensitivity independently of reductions in liver TAG/DAG/ceramide levels. Importantly, improvements in adipose and liver insulin sensitivity occurred independently of ACC phosphorylation, which we have previously shown is required for the insulin sensitizing effects of metformin [39]. These findings indicate that HIIT can uncouple inflammation and lipid metabolism from insulin sensitivity and does not depend on AMPK phosphorylation and inhibition of ACC.

Our HIIT training protocol is similar to that originally proposed by the Gibala laboratory, which involved 60 seconds of intense exercise (at 100% of VO_2 max) followed by 75 seconds of rest, repeated for 8-12 cycles, 3 times per week [60]. Importantly, this study demonstrated that the adaptations achieved in regards to muscle mitochondrial biogenesis were similar to those obtained with more than 5 times the volume of endurance (50-70% of VO_2 max) exercise training [60]. While there are higher intensity protocols that utilize less time, these protocols may not be appropriate for obese individuals with cardiometabolic

disease. And while we acknowledge that the protocol we have used is still longer than that used in many human studies, it constitutes only 90 minutes of treadmill running per week, which - to the best of our knowledge - is the lowest volume of exercise shown to have a positive effect on insulin sensitivity in obese rodents. Importantly, this volume of exercise is approximately 75% less than the majority of studies conducted in rodents which typically complete around 60 minutes of treadmill exercise per day 5 days per week [20–22,25,27,61]. In future studies, it will be interesting to titer down the work volume to determine the minimal exercise volume necessary to elicit improvements in insulin sensitivity in rodents.

Research indicates that improvements in insulin sensitivity with HIIT are comparable to [62,63], if not greater than [62,64], improvements elicited by moderate intensity continuous endurance exercise. Importantly, a number of studies have demonstrated that HIIT can improve insulin sensitivity, independent of weight loss and adiposity, in adults who are sedentary [6], overweight/obese [9] or have type 2 diabetes [7,8]. However, these studies provide little information regarding the tissue-specific mechanisms by which HIIT may improve insulin sensitivity; although it was generally assumed that the primary effects resulted from improvements in muscle insulin sensitivity. Therefore, our findings, from hyperinsulinemic-euglycemic clamps, indicating that HIIT improves adipose tissue and liver insulin sensitivity provide important information regarding the mechanisms by which HIIT improves glycemic control. Further studies in humans

using clamps are certainly warranted to investigate whether adipose tissue and liver insulin sensitivity are also improved.

Consistent with our previous findings [39], weight gain/adiposity between sedentary WT and AccDKI mice on the HFD was comparable. As intended by our selection of the exercise training program, there were no changes in body mass or adiposity compared to sedentary mice. This allowed for examination of the metabolic effects of exercise without the confounding influence of differences in adiposity. We found that despite similar adiposity and body weight, HIIT increased daily food intake in both WT and AccDKI mice, which appeared to be offset by elevations in spontaneous physical activity and subsequent increases in oxygen consumption and energy expenditure compared to sedentary controls. This is especially important since many animal studies examining effects of exercise training on insulin sensitivity [21,65,66], liver lipids [18–23,67–69] and white adipose tissue inflammation [24,25] all showed reductions in body mass, making it difficult to directly assess the effects of exercise training.

We found that our exercise training program in obese mice induced similar improvements in exercise capacity and insulin sensitivity compared to previous studies examining effects of exercise training [24,25]. In contrast to the ip ITT, the ip GTT did not show significant improvements with HIIT in either genotype, although a tendency was present in the WT mice. The reason for the lack of clear effect on glucose tolerance is likely related to the fact that we conducted an ip rather than oral GTT. Previous studies have demonstrated that exercise enhances

glucose tolerance due to IL-6 stimulation of GLP-1 [70], an effect that would not be expected to be enhanced due to the ip delivery of glucose. As we did not collect blood during the ip GTT, it is not possible to measure serum insulin or GLP-1 but we would hypothesize that insulin may have been lower in the exercise-trained mice. Future studies completing oral glucose tolerance tests and investigating the effects on this response are warranted.

Improvements in whole body insulin sensitivity following HIIT were associated with marked increases in eWAT 2-DG uptake and reductions in circulating NEFA during the hyperinsulinemic-euglycemic clamp, indicating improvements in adipose tissue insulin sensitivity. However, in contrast to previous exercise training studies, we did not detect reductions in markers of eWAT macrophage inflammation that have been linked to insulin resistance in obesity [11,71,72]. These data suggest that reductions in inflammation with exercise training reported by previous studies are most likely due to a loss of adiposity. Our studies also suggest that improvements in adipose tissue insulin sensitivity with HIIT can occur independently of reductions in inflammation. These results are in agreement with several recent studies, which have also detected a dissociation between adipose tissue inflammatory status and insulin resistance [73,74]. Due to limited quantity of tissue, we were unable to assess adipose insulin signaling in our study. Future studies examining the mechanisms by which exercise improves adipose tissue insulin sensitivity are warranted.

In addition to improving adipose tissue insulin sensitivity, we also found that HIIT improved hepatic insulin sensitivity, an effect that occurred independently of reductions in liver lipid content. Many studies in rodents [18,23,67–69] and humans [16,17,75] have indicated that exercise training can lower liver fat content. As small reductions in adiposity can have a large impact on liver fat content [76], interpreting the relative importance of exercise training on reductions in liver fat content has been difficult given that there are also reductions in adiposity in most studies. Our findings indicating that HIIT does not alter adiposity or liver fat content suggest that exercise alone is likely insufficient to lower liver lipid content. Surprisingly, despite similar lipid content, the ALT:AST ratio was lower, suggesting that liver damage induced by HFD was blunted following HIIT training. Future studies examining liver fat content and markers of liver function following HIIT training in humans are warranted.

A limitation of this study is that only one type of exercise training type was used and does not speak to the effects of other training types. HIIT is generally associated with greater use of carbohydrates rather than lipids in comparison to high-volume endurance exercise training [77]. Future studies directly comparing HIIT to classical endurance exercise training in mice would help delineate potential differences between these two types of training. Another limitation of our study was that we did not detect improvements in skeletal muscle insulin sensitivity, which may have been due to the hyperinsulinemic-euglycemic clamps being conducted 5 days after the last bout of exercise. Future studies in

which mice are clamped 72 hrs after the last exercise bout may be important to avoid detraining effects in muscle.

In summary, HIIT improved exercise capacity and whole-body glucose homeostasis by enhancing liver and adipose tissue insulin sensitivity. These effects were independent of reductions in adiposity/adipose tissue cell size indicating the therapeutic potential of exercise independent of weight loss. Improved insulin sensitivity was also independent of adipose tissue inflammation, macrophage infiltration/inflammation or reductions in liver lipid content indicating dissociation between these parameters and insulin resistance. Lastly, we demonstrated that in contrast to metformin, HIIT exercise training improves insulin sensitivity independently of the AMPK-ACC signaling pathway. Future studies identifying the mechanism by which HIIT improves insulin sensitivity may reveal novel strategies to improve glucose homeostasis in individuals with type 2 diabetes.

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AUTHOR CONTRIBUTIONS: KM, SRS, MF and GRS conception and design of research; KM, SS, MCS, and MDF performed experiments; KM and GRS analyzed data; KM and GRS interpreted results of experiments; KM and GRS prepared figures; KM and GRS drafted manuscript; KM, BEK, MDF and GRS edited and revised manuscript; KM, SRS, BEK, MCS, MDF and GRS approved final version of manuscript. KM and GRS are responsible for the integrity of the work as a whole.

References

[1] Tuomilehto, J., Lindström, J., Eriksson, J.G., Valle, T.T., Hämäläinen, H., Ilanne-Parikka, P., et al., 2001. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. The New England Journal of Medicine 344(18): 1343–50, Doi: 10.1056/NEJM200105033441801.

[2] Knowler, W.C., Barrett-Connor, E., Fowler, S.E., Hamman, R.F., Lachin, J.M., Walker, E.A., et al., 2002. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. The New England Journal of Medicine 346(6): 393–403, Doi: 10.1056/NEJMoa012512.

[3] Pan, X.R., Li, G.W., Hu, Y.H., Wang, J.X., Yang, W.Y., An, Z.X., et al., 1997. Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance: The Da Qing IGT and diabetes study. Diabetes Care 20(4): 537–44, Doi: 10.2337/diacare.20.4.537.

[4] Godin, G., Desharnais, R., Valois, P., Lepage, L., Jobin, J., Bradet, R., 1994. Differences in perceived barriers to exercise between high and low intenders: Observations among different populations. American Journal of Health Promotion 8(4): 279–85, Doi: 10.4278/0890-1171-8.4.279.

[5] Marquis-Gravela, G., Hayamia, D., Juneaua, M., Nigama, A., Guilbeaulta, V., Latoura, E., et al., 2015. Intensive lifestyle intervention including high-intensity interval training program improves insulin resistance and fasting plasma glucose in obese patients. Preventive Medicine Reports 2: 314–8, Doi: 10.1016/j.pmedr.2015.04.015.

[6] Cocks, M., Shepherd, S., Tipton, K., Wagenmakers, A., Shaw, C., 2010. High-intensity interval training improves microvascular and macrovascular function and insulin sensitivity. Br J Sports Med 44(i9), Doi: 10.1136/bjsm.2010.078972.27.

[7] Shaban, N., Kenno, K.A., Milne, K.J., 2014. The effects of a 2 week modified high intensity interval training program on the homeostatic model of insulin resistance (HOMA-IR) in adults with type 2 diabetes. The Journal of Sports Medicine and Physical Fitness 54(2): 203–9.

[8] Little, J.P., Gillen, J.B., Percival, M.E., Safdar, A., Tarnopolsky, M. a, Punthakee, Z., et al., 2011. Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in

patients with type 2 diabetes. Journal of Applied Physiology (Bethesda, Md. : 1985) 111(6): 1554–60, Doi: 10.1152/japplphysiol.00921.2011.

[9] Cocks, M., Shaw, C., Shepherd, S., Fisher, J., Ranasinghe, A., Barker, T., et al., 2015. Sprint interval and moderate-intensity continuous training have equal benefits on aerobic capacity, insulin sensitivity, muscle capillarisation and endothelial eNOS/NAD(P)Hoxidase. J Physiol 10.1113/jp, Doi: 10.1113/jphysiol.2014.285254.

[10] Lumeng, C.N., Delproposto, J.B., Westcott, D.J., Saltiel, A.R., 2008. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. Diabetes 57(12): 3239–46, Doi: 10.2337/db08-0872.

[11] Lumeng, C.N., DeYoung, S.M., Bodzin, J.L., Saltiel, A.R., 2007. Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. Diabetes 56(1): 16–23, Doi: 10.2337/db06-1076.

[12] Fabbrini, E., Sullivan, S., Klein, S., 2010. Obesity and nonalcoholic fatty liver disease: Biochemical, metabolic, and clinical implications. Hepatology 51(2): 679–89, Doi: 10.1002/hep.23280.

[13] Pagadala, M.R., McCullough, A.J., 2012. Non-alcoholic fatty liver disease and obesity: not all about body mass index. The American Journal of Gastroenterology 107(12): 1859–61, Doi: 10.1038/ajg.2012.320.

[14] Heilbronn, L.K., Campbell, L. V., 2008. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. Current Pharmaceutical Design 14(12): 1225–30, Doi: 10.2174/138161208784246153.

[15] Lee, J., 2013. Adipose tissue macrophages in the development of obesity-induced inflammation, insulin resistance and type 2 Diabetes. Archives of Pharmacal Research: 208–22, Doi: 10.1007/s12272-013-0023-8.

[16] Zelber-Sagi, S., Nitzan-Kaluski, D., Goldsmith, R., Webb, M., Zvibel, I., Goldiner, I., et al., 2008. Role of leisure-time physical activity in nonalcoholic fatty liver disease: a population-based study. Hepatology (Baltimore, Md.) 48(6): 1791–8, Doi: 10.1002/hep.22525.

[17] Johnson, N.A., Sachinwalla, T., Walton, D.W., Smith, K., Armstrong, A., Thompson, M.W., et al., 2009. Aerobic exercise training reduces hepatic and visceral lipids in obese individuals without weight loss. Hepatology 50(4): 1105–12, Doi: 10.1002/hep.23129.

[18] Jordy, A.B., Kraakman, M.J., Gardner, T., Estevez, E., Kammoun, H.L., Weir, J.M., et al., 2015. Analysis of the liver lipidome reveals insights into the protective effect of exercise on high-fat diet-induced hepatosteatosis in mice. Am J Physiol Endocrinol Metab 308(9): E778–91, Doi: 10.1152/ajpendo.00547.2014.

[19] Cintra, D.E., Ropelle, E.R., Vitto, M.F., Luciano, T.F., Souza, D.R., Engelmann, J., et al., 2012. Reversion of hepatic steatosis by exercise training in obese mice: The role of sterol regulatory element-binding protein-1c. Life Sciences 91(11-12): 395–401, Doi: 10.1016/j.lfs.2012.08.002.

[20] Cho, J., Lee, I., Kim, D. et al., 2014. Effect of aerobic exercise training on non-alcoholic fatty liver disease induced by a high fat diet in C57BL/6 mice. J Exerc Nutrition Biochem 18(4): 339–46, Doi: 10.5717/jenb.2014.18.4.339.

[21] Marques, C.M.M., Motta, V.F., Torres, T.S., Aguila, M.B., Mandarim-de-Lacerda, C.A., 2010. Beneficial effects of exercise training (treadmill) on insulin resistance and nonalcoholic fatty liver disease in high-fat fed C57BL/6 mice. Braz J Med Biol Res 43(5): 467–75.

[22] Berglund, E.D., Lustig, D.G., Baheza, R.A., Hasenour, C.M., Lee-Young, R.S., Donahue, E.P., et al., 2011. Hepatic glucagon action is essential for exercise-induced reversal of mouse fatty liver. Diabetes 60(11): 2720–9, Doi: 10.2337/db11-0455.

[23] Haram, P.M., Kemi, O.J., Lee, S.J., Bendheim, M., Al-Share, Q.Y., Waldum, H.L., et al., 2009. Aerobic interval training vs. continuous moderate exercise in the metabolic syndrome of rats artificially selected for low aerobic capacity. Cardiovascular Research 81(4): 723–32, Doi: 10.1093/cvr/cvn332.

[24] Bradley, R.L., Jeon, J.Y., Liu, F.-F., Maratos-Flier, E., 2008. Voluntary exercise improves insulin sensitivity and adipose tissue inflammation in diet-induced obese mice. American Journal of Physiology. Endocrinology and Metabolism 295(3): E586–94, Doi: 10.1152/ajpendo.00309.2007.

[25] Vieira, V.J., Valentine, R.J., Wilund, K.R., Antao, N., Baynard, T., Woods, J.A., 2009. Effects of exercise and low-fat diet on adipose tissue inflammation and metabolic complications in obese mice. American Journal of Physiology. Endocrinology and Metabolism 296(5): E1164–71, Doi: 10.1152/ajpendo.00054.2009.

[26] Vieira, V.J., Valentine, R.J., Wilund, K.R., Antao, N., Baynard, T., Woods, J.A., 2009. Effects of exercise and low-fat diet on adipose tissue inflammation and metabolic complications in obese mice. American Journal of Physiology. Endocrinology and Metabolism 296(5): E1164–71, Doi: 10.1152/ajpendo.00054.2009.

[27] Kawanishi, N., Yano, H., Yokogawa, Y., Suzuki, K., 2010. Exercise training inhibits inflammation in adipose tissue via both suppression of macrophage infiltration and acceleration of phenotypic switching from M1 to M2 macrophages in high-fat-diet-induced obese mice. Exercise Immunology Review 16: 105–18.

[28] Gibala, M.J., McGee, S.L., Garnham, A.P., Howlett, K.F., Snow, R.J., Hargreaves, M., 2009. Brief intense interval exercise activates AMPK and p38 MAPK signaling and increases the expression of PGC-1alpha in human skeletal muscle. Journal of Applied Physiology (Bethesda, Md. : 1985) 106(3): 929–34, Doi: 10.1152/japplphysiol.90880.2008.

[29] Stephens, T.J., Chen, Z.-P., Canny, B.J., Michell, B.J., Kemp, B.E., McConell, G.K., 2002. Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise. American Journal of Physiology. Endocrinology and Metabolism 282(3): E688–94, Doi: 10.1152/ajpendo.00101.2001.

[30] Park, H., Kaushik, V.K., Constant, S., Prentki, M., Przybytkowski, E., Ruderman, N., et al., 2002. Coordinate regulation of malonyl-CoA decarboxylase, sn-glycerol-3-phosphate acyltransferase, and acetyl-CoA carboxylase by AMP-activated protein kinase in rat tissues in response to exercise. Journal of Biological Chemistry 277(36): 32571–7, Doi: 10.1074/jbc.M201692200.

[31] Wojtaszewski, J.F., Nielsen, P., Hansen, B.F., Richter, E.A., Kiens, B., 2000. Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle. The Journal of Physiology 528(Pt 1): 221–6, Doi: 10.1111/j.1469-7793.2000.t01-1-00221.x.

[32] Camacho, R.C., Donahue, E.P., James, F.D., Berglund, E.D., Wasserman, D.H., 2005. Energy State of the Liver During Short Term and Exhaustive Exercise in C57BL/6J Mice. Am.J.Physiol Endocrinol.Metab 290(3): E405–8.

[33] Watt, M.J., Holmes, A.G., Pinnamaneni, S.K., Garnham, A.P., Steinberg, G.R., Kemp, B.E., et al., 2006. Regulation of HSL serine phosphorylation

in skeletal muscle and adipose tissue. American Journal of Physiology. Endocrinology and Metabolism 290(3): E500–8, Doi: 10.1152/ajpendo.00361.2005.

[34] Koh, H.-J., Hirshman, M.F., He, H., Li, Y., Manabe, Y., Balschi, J.A., et al., 2007. Adrenaline is a critical mediator of acute exercise-induced AMP-activated protein kinase activation in adipocytes. The Biochemical Journal 403(3): 473–81, Doi: 10.1042/BJ20061479.

[35] Galic, S., Fullerton, M.D., Schertzer, J.D., Sikkema, S., Marcinko, K., Walkley, C.R., et al., 2011. Hematopoietic AMPK β1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. Journal of Clinical Investigation 121(12): 4903–15, Doi: 10.1172/JCI58577.

[36] Sag, D., Carling, D., Stout, R.D., Suttles, J., 2008. Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. Journal of Immunology (Baltimore, Md. : 1950) 181(12): 8633–41, Doi: 181/12/8633 [pii].

[37] Steinberg, G.R., Schertzer, J.D., 2014. AMPK promotes macrophage fatty acid oxidative metabolism to mitigate inflammation: implications for diabetes and cardiovascular disease. Immunology and Cell Biology 92(4): 340–5, Doi: 10.1038/icb.2014.11.

[38] Mounier, R., Théret, M., Arnold, L., Cuvellier, S., Bultot, L., Göransson, O., et al., 2013. AMPKα1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration. Cell Metabolism 18(2): 251–64, Doi: 10.1016/j.cmet.2013.06.017.

[39] Fullerton, M.D., Galic, S., Marcinko, K., Sikkema, S., Pulinilkunnil, T., Chen, Z.-P., et al., 2013. Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. Nature Medicine 19(12): 1649–54, Doi: 10.1038/nm.3372.

[40] Steinberg, G.R., Jorgensen, S.B., 2007. The AMP-activated protein kinase: role in regulation of skeletal muscle metabolism and insulin sensitivity. Mini Rev Med Chem 7(5): 519–26, Doi: 10.2174/138955707780619662.

[41] Crane, J.D., Palanivel, R., Mottillo, E.P., Bujak, A.L., Wang, H., Ford, R.J., et al., 2015. Inhibiting peripheral serotonin synthesis reduces obesity and metabolic dysfunction by promoting brown adipose tissue thermogenesis. Nature Medicine 21: 166–72, Doi: 10.1038/nm.3766.

[42] Watt, M.J., Dzamko, N., Thomas, W.G., Rose-John, S., Ernst, M., Carling, D., et al., 2006. CNTF reverses obesity-induced insulin resistance by activating skeletal muscle AMPK. Nature Medicine 12(5): 541–8, Doi: 10.1038/nm1383.

[43] Steele, R., 1959. Influences of glucose loading and of injected insulin on hepatic glucose output. Ann N Y Acad Sci 82: 420–30, Doi: 10.1111/j.1749-6632.1959.tb44923.x.

[44] Bligh, E.G., Dyer, W.J., 1959. A Rapid Method of Total Lipid Extraction and Purification. Can. J. Biochem. Physiol. 37(8): 911–7, Doi: 10.1139/o59-099.

[45] Preiss, J., Loomis, C.R., Bishop, W.R., Stein, R., Niedel, J.E., Bell, R.M., 1986. Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and ras- and sis-transformed normal rat kidney cells. Journal of Biological Chemistry 261(19): 8597–600.

[46] Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.) 25(4): 402–8, Doi: 10.1006/meth.2001.1262.

[47] Lund, J., Hafstad, A.D., Boardman, N.T., Rossvoll, L., Rolim, N.P., Ahmed, M.S., et al., 2015. Exercise training promotes cardioprotection through oxygen-sparing action in high fat-fed mice. Am J Physiol Heart and Circulatory Physiology 308(8): H823–9, Doi: 10.1152/ajpheart.00734.2014.

[48] Apontes, P., Liu, Z., Su, K., Benard, O., Youn, D.Y., Li, X., et al., 2014. Mangiferin Stimulates Carbohydrate Oxidation and Protects against High Fat Diet Induced Metabolic Disorders. Diabetes 63(11): 3626–36, Doi: 10.2337/db14-0006.

[49] Corpeleijn, E., Saris, W.H.M., Blaak, E.E., 2009. Metabolic flexibility in the development of insulin resistance and type 2 diabetes: Effects of lifestyle: Etiology and Pathophysiology. Obesity Reviews 10(2): 178–93, Doi: 10.1111/j.1467-789X.2008.00544.x.

[50] Jorgensen, S.B., O'Neill, H.M., Sylow, L., Honeyman, J., Hewitt, K.A., Palanivel, R., et al., 2013. Deletion of skeletal muscle SOCS3 prevents insulin resistance in obesity. Diabetes 62(1): 56–64, Doi: 10.2337/db12-0443.

[51] Shulman, G.I., 1999. Cellular mechanisms of insulin resistance in humans. The American Journal of Cardiology 84(1A): 3J – 10J, Doi: 10.1016/S0002-9149(99)00350-1.

[52] Yu, C., Chen, Y., Cline, G.W., Zhang, D., Zong, H., Wang, Y., et al., 2002. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. Journal of Biological Chemistry 277(52): 50230–6, Doi: 10.1074/jbc.M200958200.

[53] O'Neill, H.M., Lally, J.S., Galic, S., Thomas, M., Azizi, P.D., Fullerton, M.D., et al., 2014. AMPK phosphorylation of ACC2 is required for skeletal muscle fatty acid oxidation and insulin sensitivity in mice. Diabetologia 57(8): 1693–702, Doi: 10.1007/s00125-014-3273-1.

[54] Nagasawa, J., Sato, Y., Ishiko, T., 1990. Effect of training and detraining on in vivo insulin sensitivity. International Journal of Sports Medicine 11(2): 107–10, Doi: 10.1055/s-2007-1024772.

[55] Chi, M.M., Hintz, C.S., Coyle, E.F., Martin, W.H., Ivy, J.L., Nemeth, P.M., et al., 1983. Effects of detraining on enzymes of energy metabolism in individual human muscle fibers. The American Journal of Physiology 244(3): C276–87.

[56] Mazzucatto, F., Higa, T., Fonseca-Alaniz, M., Evangelista, F., 2014. Reversal of metabolic adaptations induced by physical training after two weeks of physical detraining. Int J Clin Exp Med 7(8): 2000–8.

[57] Nogueira, L., Ramirez-Sanchez, I., Perkins, G.A., Murphy, A., Taub, P.R., Ceballos, G., et al., 2011. (-)-Epicatechin enhances fatigue resistance and oxidative capacity in mouse muscle. The Journal of Physiology 589(Pt 18): 4615–31, Doi: 10.1113/jphysiol.2011.209924.

[58] Peirce, V., Vidal-Puig, A., 2013. Regulation of glucose homoeostasis by brown adipose tissue. The Lancet Diabetes & Endocrinology 1(4): 353–60, Doi: 10.1016/S2213-8587(13)70055-X.

[59] Boström, P., Wu, J., Jedrychowski, M.P., Korde, A., Ye, L., Lo, J.C., et al., 2012. A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature 481(7382): 463–8, Doi: 10.1038/nature10777.

[60] Little, J.P., Safdar, A., Wilkin, G.P., Tarnopolsky, M.A., Gibala, M.J., 2010. A practical model of low-volume high-intensity interval training

induces mitochondrial biogenesis in human skeletal muscle: potential mechanisms. The Journal of Physiology 588(Pt 6): 1011–22, Doi: 10.1113/jphysiol.2009.181743.

[61] Motta, V.F., de Lacerda, C.A., 2012. Beneficial Effects of Exercise Training (Treadmill) on Body Mass and Skeletal Muscle Capillaries/Myocyte Ratio in C57BL/6 Mice Fed High-Fat Diet. INTERNATIONAL JOURNAL OF MORPHOLOGY 30(1): 205–10.

[62] Mitranun, W., Deerochanawong, C., Tanaka, H., Suksom, D., 2014. Continuous vs interval training on glycemic control and macro- and microvascular reactivity in type 2 diabetic patients. Scand J Med Sci Sports 24: e69–76, Doi: 10.1111/sms.12112.

[63] Nybo, L., Sundstrup, E., Jakobsen, M.D., Mohr, M., Hornstrup, T., Simonsen, L., et al., 2010. High-intensity training versus traditional exercise interventions for promoting health. Medicine and Science in Sports and Exercise 42(10): 1951–8, Doi: 10.1249/MSS.0b013e3181d99203.

[64] Tjønna, A.E., Lee, S.J., Rognmo, Ø., Stølen, T.O., Bye, A., Haram, P.M., et al., 2008. Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: A pilot study. Circulation 118(4): 346–54, Doi: 10.1161/CIRCULATIONAHA.108.772822.

[65] Rao, X., Zhong, J., Xu, X., Jordan, B., Maurya, S., Braunstein, Z., et al., 2013. Exercise protects against diet-induced insulin resistance through downregulation of protein kinase Cβ in mice. PLoS ONE 8(12), Doi: 10.1371/journal.pone.0081364.

[66] Sarvas, J., Otis, J., Khaper, N., Lees, S., 2015. Voluntary physical activity prevents insulin resistance in a tissue specific manner. Physiol Rep 3(2): e12277, Doi: 10.14814/phy2.12277.

[67] Rector, R.S., Thyfault, J.P., Morris, R.T., Laye, M.J., Borengasser, S.J., Booth, F.W., et al., 2008. Daily exercise increases hepatic fatty acid oxidation and prevents steatosis in Otsuka Long-Evans Tokushima Fatty rats. American Journal of Physiology. Gastrointestinal and Liver Physiology 294(3): G619–26, Doi: 10.1152/ajpgi.00428.2007.

[68] Rector, R.S., Thyfault, J.P., Laye, M.J., Morris, R.T., Borengasser, S.J., Uptergrove, G.M., et al., 2008. Cessation of daily exercise dramatically alters precursors of hepatic steatosis in Otsuka Long-Evans Tokushima

Fatty (OLETF) rats. The Journal of Physiology 586(Pt 17): 4241–9, Doi: 10.1113/jphysiol.2008.156745.

[69] Thyfault, J.P., Rector, R.S., Uptergrove, G.M., Borengasser, S.J., Morris, E.M., Wei, Y., et al., 2009. Rats selectively bred for low aerobic capacity have reduced hepatic mitochondrial oxidative capacity and susceptibility to hepatic steatosis and injury. The Journal of Physiology 587(Pt 8): 1805–16, Doi: 10.1113/jphysiol.2009.169060.

[70] Ellingsgaard, H., Hauselmann, I., Schuler, B., Habib, A.M., Baggio, L.L., Meier, D.T., et al., 2011. Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells. Nature Medicine: 1481–9, Doi: 10.1038/nm.2513.

[71] Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L., Ferrante, A.W., 2003. Obesity is associated with macrophage accumulation in adipose tissue. Journal of Clinical Investigation 112(12): 1796–808, Doi: 10.1172/JCI200319246.

[72] Pedersen, B.K., 2006. The anti-inflammatory effect of exercise: its role in diabetes and cardiovascular disease control. Essays Biochem 42: 105–17.

[73] Turner, N., Kowalski, G.M., Leslie, S.J., Risis, S., Yang, C., Lee-Young, R.S., et al., 2013. Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding. Diabetologia 56(7): 1638–48, Doi: 10.1007/s00125-013-2913-1.

[74] Aparicio-Vergara, M., Hommelberg, P.P.H., Schreurs, M., Gruben, N., Stienstra, R., Shiri-Sverdlov, R., et al., 2013. Tumor necrosis factor receptor 1 gain-of-function mutation aggravates nonalcoholic fatty liver disease but does not cause insulin resistance in a murine model. Hepatology (Baltimore, Md.) 57(2): 566–76, Doi: 10.1002/hep.26046.

[75] Hallsworth, K., Fattakhova, G., Hollingsworth, K.G., Thoma, C., Moore, S., Taylor, R., et al., 2011. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. Gut 60(9): 1278–83, Doi: 10.1136/gut.2011.242073.

[76] Petersen, K.F., Dufour, S., Befroy, D., Lehrke, M., Hendler, R.E., Shulman, G.I., 2005. Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. Diabetes 54(3): 603–8, Doi: 10.2337/diabetes.54.3.603.

[77] Romijn, J.A., Gastaldelli, A., Horowitz, J.F., Endert, E., Wolfe, R.R., 1993. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. The American Journal of Physiology 265(3p1): 380–91.

Table Legends

Table 1: Analysis of tissue weights. Data are expressed as means ± SEM.

Table 2: Serum cytokines in HFD Sedentary and HFD Exercised mice. Data are expressed as means ± SEM, $^{***}p<0.001$, for difference between WT sedentary vs WT exercise; $^{§§}p<0.01$, for difference from WT vs AccDKI, as determined by two-way ANOVA and Bonferonni *post hoc* test.

Table 3: Analysis of liver inflammatory markers. Data are expressed as means ± SEM, $^{†}p<0.05$, $^{†††}p<0.001$, for difference from sedentary vs exercise, as determined by two-way ANOVA and Bonferonni *post hoc* test.

Figure Legends

Figure 1. HIIT increases exercise capacity and metabolic flexibility independent of body weight and adiposity. (**A**) Body weights of WT and AccDKI mice that were sedentary or exercise trained (two-way repeated measures ANOVA). (**B**) Whole-body percent adiposity, with representative CT scan images. Areas highlighted in red represent adipose region. (**C**) HIIT time to exhaustion, (**D**) HIIT speed at exhaustion, and (**E**) HIIT distance traversed. (**F**) Average daily food intake. (**G**) Average daily ambient activity. (**H**) Average daily energy expenditure. (**I**) Average daily VO_2 consumption and VO_2 production. (**J**) Basal VO_2 consumption (**K**) Daily average RER. (**L**) Daily average CHO oxidation. (**M**) Daily average lipid oxidation. Number of mice are denoted on the graph. Data are expressed as means ± SEM, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$, for difference from sedentary vs exercise, as determined by two-way ANOVA and Bonferonni *post hoc* test.

Figure 2. HIIT improves insulin sensitivity independent of AMPK phosphorylation of ACC. (**A**) Fed blood glucose concentration. (**B**) 12 hour fasting blood glucose concentration and (**C**) serum insulin concentration. (**D**) ITT (ip) comparison between WT sedentary and WT exercise trained mice. (**E**) ITT (ip) comparison between AccDKI sedentary and AccDKI exercise trained mice. (**F**) ITT (ip) AUC. (**G**) GTT (ip) comparison between WT sedentary and WT exercise trained mice. (**H**) GTT (ip) comparison between AccDKI sedentary and AccDKI exercise trained mice. (**I**) GTT (ip) AUC. Number of mice is denoted on

the graph. Data are expressed as means ± SEM, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, for difference from sedentary vs exercise, as determined by two-way ANOVA and Bonferonni *post hoc* test. *p<0.05, and **p<0.01 as a comparison between sedentary and exercise, as determined by two-way repeated measures ANOVA and Bonferonni *post hoc* test.

Figure 3. HIIT improves insulin sensitivity independently of skeletal muscle insulin sensitivity. (**A**) Clamped glucose infusion rate (GIR) and (**B**) glucose disposal rate (GDR). (**C**) Skeletal muscle 2-DG uptake, (**D**) TAG, (**E**) DAG and ceramide accumulation. (**F**) Skeletal muscle pAkt Thr308/Akt total, pAkt Ser473/Akt total and pAS160 Thr642/GAPDH at the completion of the clamp (from separate gels). **(G)** Protein expression of Complex V, Complex IV, Complex III and Complex II/GAPDH of the electron transport chain and **(H)** PGC1α/GAPDH in skeletal muscle relative to HFD WT. Number of mice are denoted on the graph. Data are expressed as means ± SEM, $^{\dagger\dagger}p<0.01$ for difference from sedentary vs exercise, $^{\P}p<0.05$ for difference between WT and AccDKI, as determined by two-way ANOVA and Bonferonni *post hoc* test.

Figure 4. HIIT increases adipose tissue glucose uptake independent of ACC phosphorylation but does not alter inflammation, macrophage infiltration or browning markers. (**A**) Adipose tissue 2-DG uptake. (**B**) Clamped circulating non-esterified free fatty acid levels. (**C**) Adipocyte size. (**D**) Adipose tissue *Cd68* and *Emr1* mRNA expression. (**E**) F4/80+ cells in adipose tissue. (**F**) Adipose

tissue inflammatory markers *Il6*, *Tnfa*, and *Kc*. (**G**) Adipose tissue macrophage *arginase* and *Nos2* mRNA expression. (**H**) Adipose tissue macrophage *Tnfa, Il1b*, and *Kc* mRNA expression. (**I**) Adipose tissue *Cidea, Prdm16, Pdk4*, *Pgc1a*, and *Glut4* mRNA expression.

Figure 5. HIIT improves hepatic insulin sensitivity independent of ACC phosphorylation and liver triglycerides. (**A**) HGP and (**B**) percent suppression of insulin. (**C**) Relative expression of liver gluconeogenic gene expression of *G6pase* and *Pck1*. (**D**) Liver TAG content. (**E**) Liver DAG, and ceramide content and representative liver H&E images. (**F**) Liver area of fibrosis and representative liver trichrome stain images. Fibrosis represented by collagen is stained in blue. (**G**) Liver glycogen content. **(H**) Serum ALT and AST concentrations. (**I**) ALT/AST ratio. Number of mice is denoted on the graphs. Data are expressed as means ± SEM, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ for difference from sedentary vs exercise, as determined by two-way ANOVA and Bonferonni *post hoc* test.

Supplementary Figure 1. (**A**) Acute pAMPK Thr172 to AMPK total and (**B**) pACC Ser79/212 to ACC total in liver after an acute bout of exercise. (**C**) Clamped GIR curve comparison between WT sedentary and WT exercise trained mice during clamp. (**D**) Clamped GIR curve comparison between AccDKI sedentary and AccDKI exercise trained mice during clamp. (**E**) Blood glucose comparison between WT sedentary and WT exercise trained mice during clamp.

(**F**) Blood glucose comparison between AccDKI sedentary and AccDKI exercise trained mice during clamp. Number of mice is denoted on the graph. Data are expressed as means ± SEM, *$p<0.05$ for difference between sedentary vs exercise as determined by t-test.

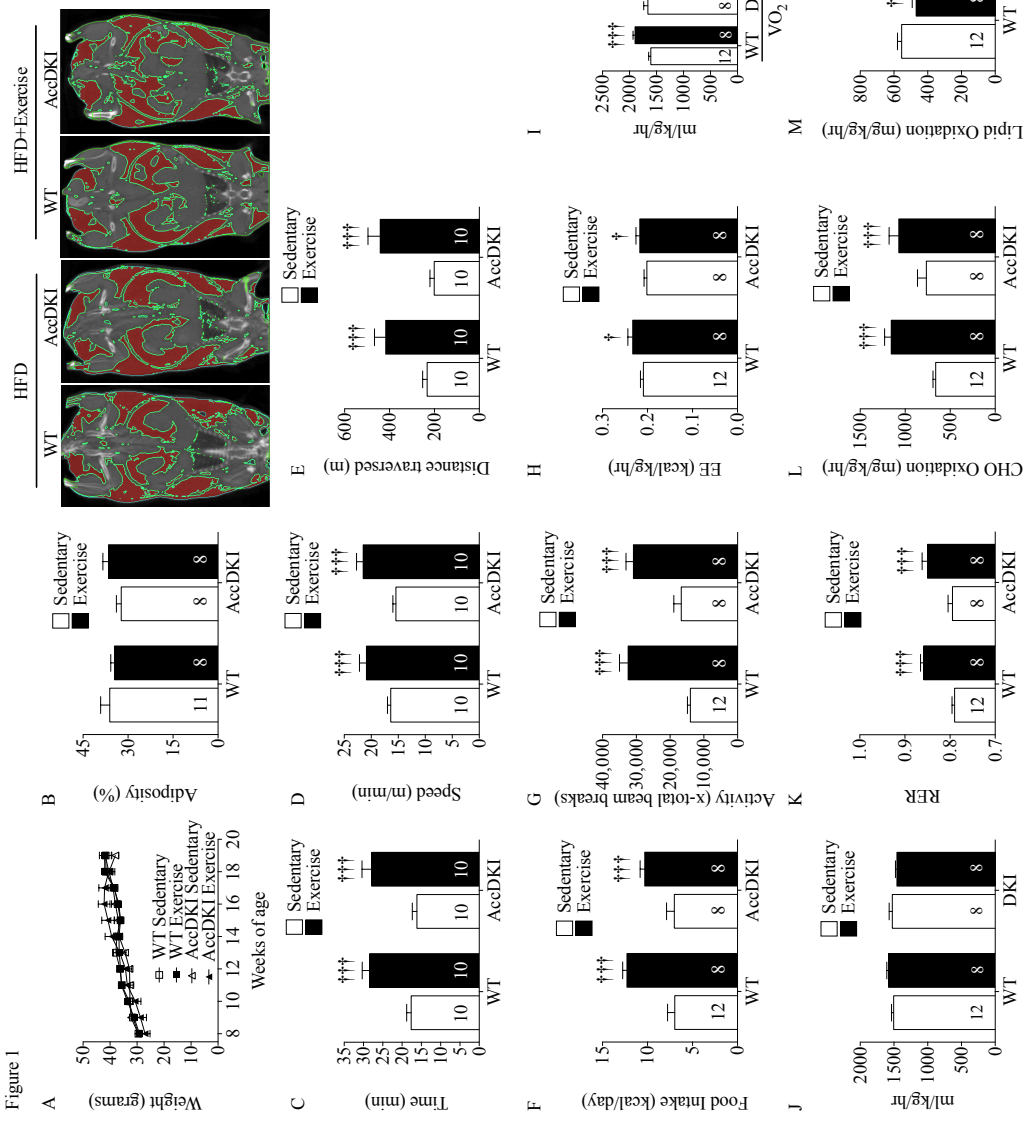


Figure 1
A
Weight (grams)
Weeks of age
WT Sedentary
WT Exercise
AccDKI Sedentary
AccDKI Exercise
B
Adiposity (%)
HFD
HFD+Exercise
WT
AccDKI
Sedentary
Exercise
C
Time (min)
D
Speed (m/min)
E
Distance traversed (m)
F
Food Intake (kcal/day)
G
Activity (x-total beam breaks)
H
EE (kcal/kg/hr)
I
ml/kg/hr
VO2
VCO2
DKI
J
ml/kg/hr
K
RER
L
CHO Oxidation (mg/kg/hr)
M
Lipid Oxidation (mg/kg/hr)

Figure 2

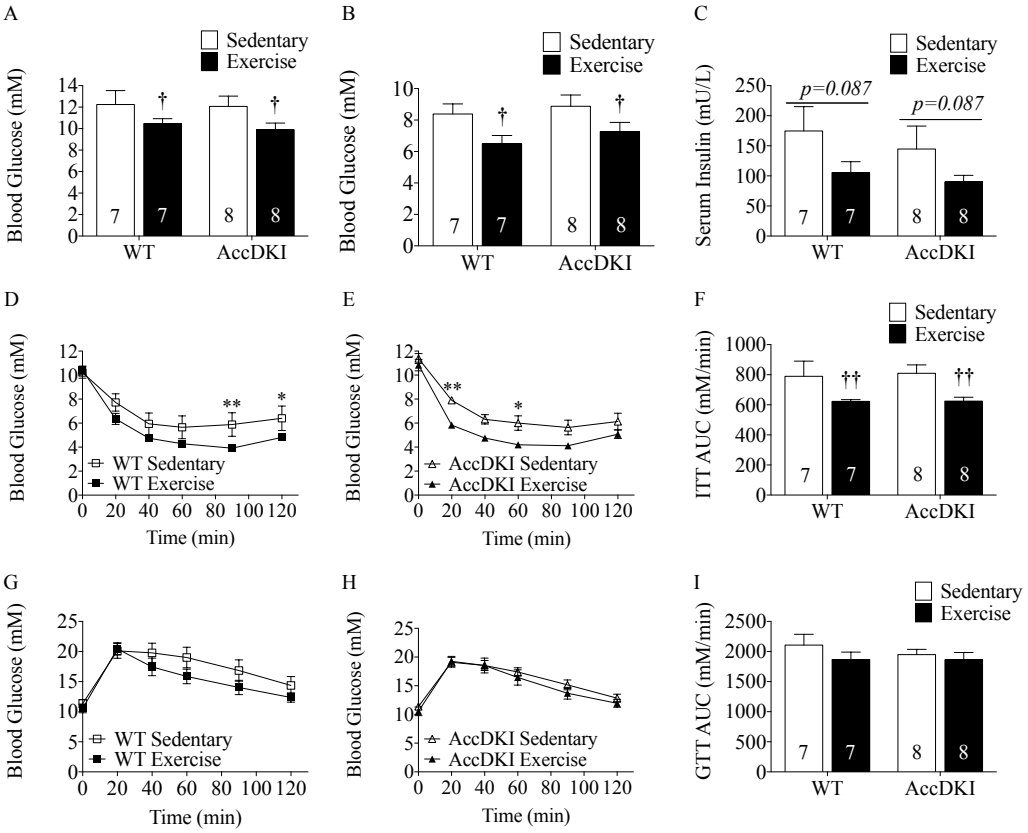
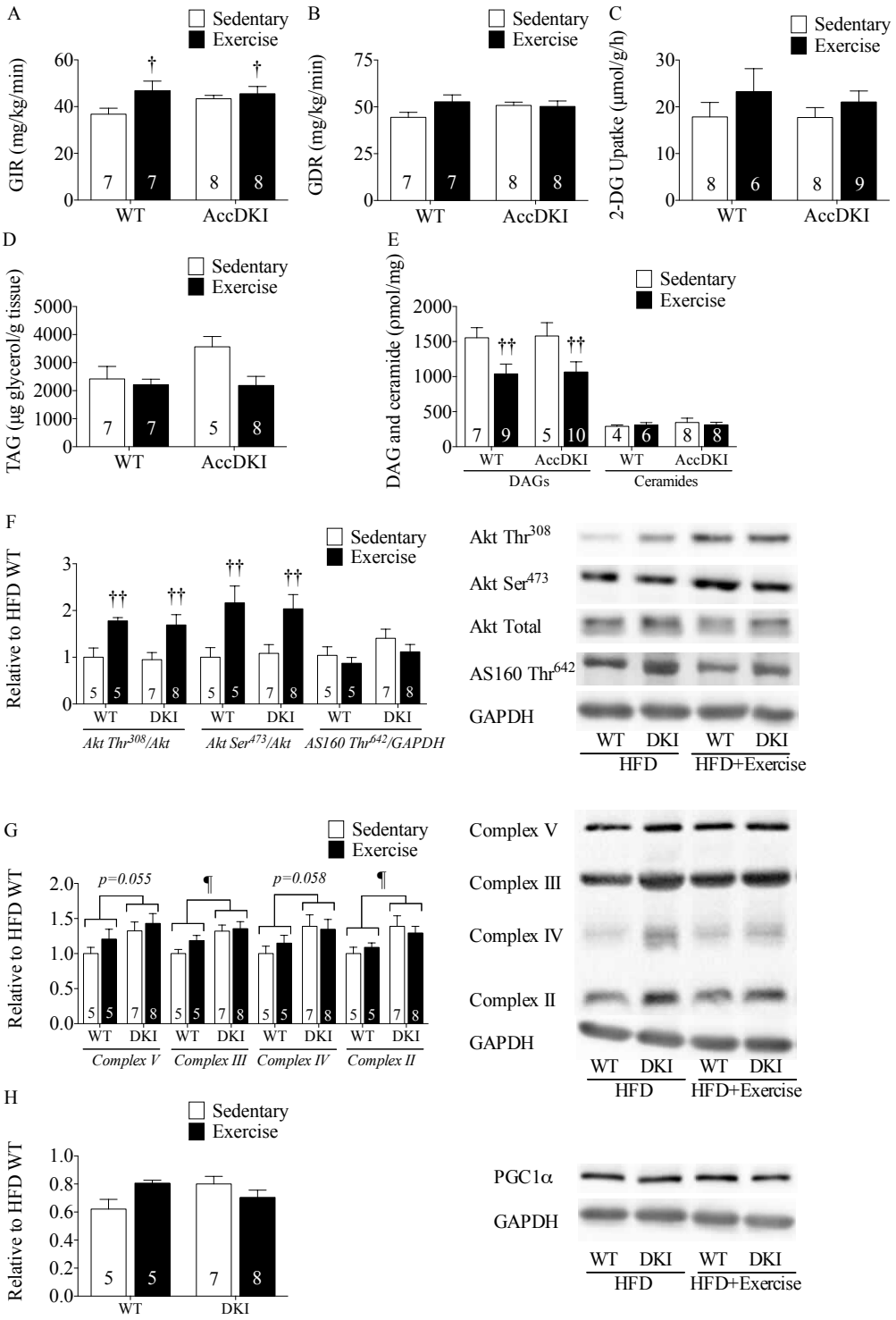

A
Sedentary
Exercise
Blood Glucose (mM)
WT
AccDKI
B
Blood Glucose (mM)
C
Serum Insulin (mU/L)
p=0.087
p=0.087
D
Blood Glucose (mM)
WT Sedentary
WT Exercise
Time (min)
E
Blood Glucose (mM)
AccDKI Sedentary
AccDKI Exercise
Time (min)
F
ITT AUC (mM/min)
G
Blood Glucose (mM)
WT Sedentary
WT Exercise
Time (min)
H
Blood Glucose (mM)
AccDKI Sedentary
AccDKI Exercise
Time (min)
I
GTT AUC (mM/min)

Figure 3


A
GIR (mg/kg/min)
Sedentary
Exercise
WT
AccDKI
B
GDR (mg/kg/min)
C
2-DG Upatke (µmol/g/h)
D
TAG (µg glycerol/g tissue)
E
DAG and ceramide (pmol/mg)
DAGs
Ceramides
F
Relative to HFD WT
Akt Thr308/Akt
Akt Ser473/Akt
AS160 Thr642/GAPDH
Akt Thr308
Akt Ser473
Akt Total
AS160 Thr642
GAPDH
HFD
HFD+Exercise
G
p=0.055
p=0.058
Complex V
Complex III
Complex IV
Complex II
H
PGC1α

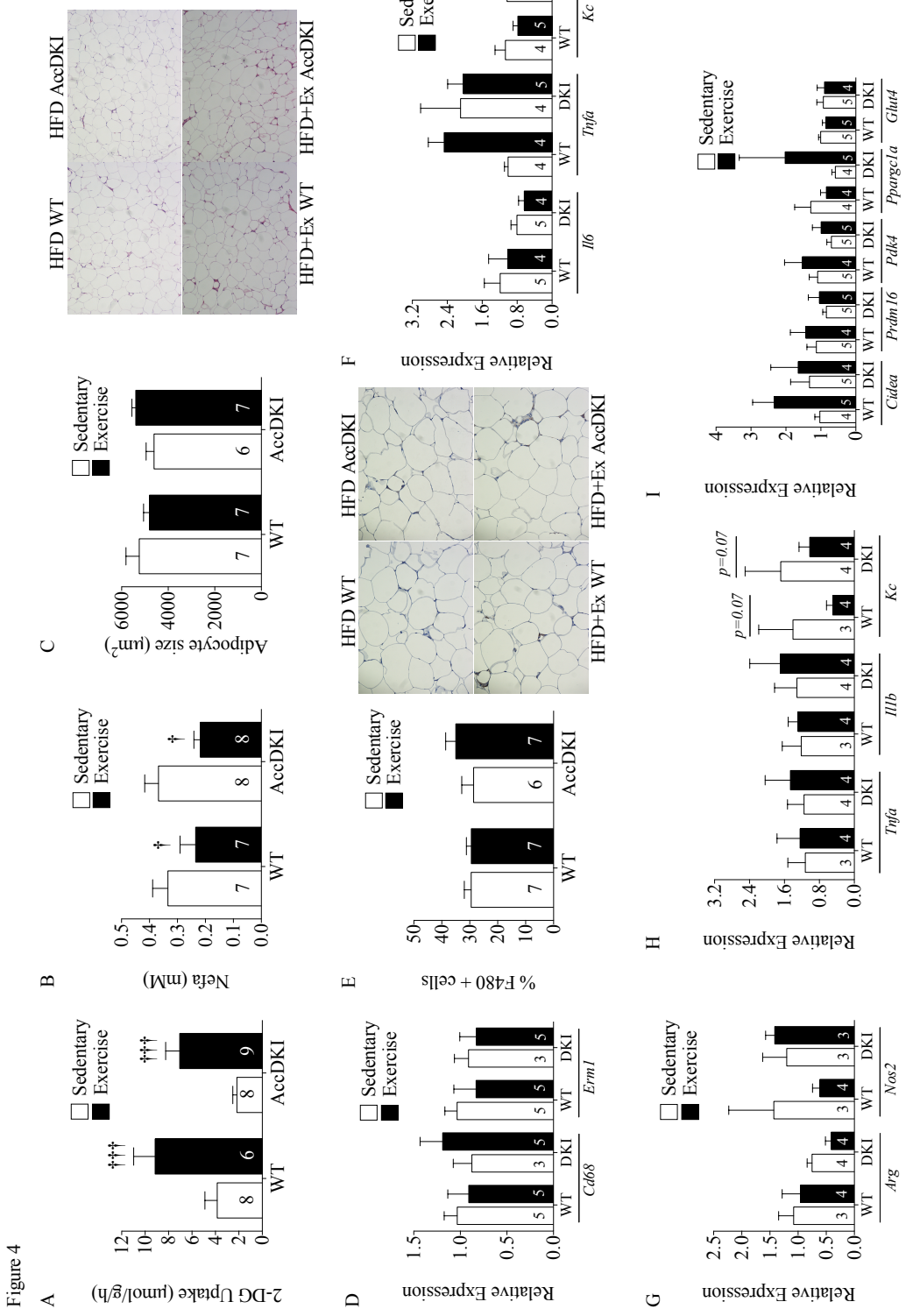


Figure 4
A
2-DG Uptake (μmol/g/h)
Sedentary
Exercise
WT
AccDKI
B
Nefa (mM)
C
Adipocyte size (μm²)
HFD WT
HFD AccDKI
HFD+Ex WT
HFD+Ex AccDKI
D
Relative Expression
Cd68
Erm1
E
% F480 + cells
F
Il6
Tnfa
Kc
G
Arg
Nos2
H
Il1b
p=0.07
I
Cidea
Prdm16
Pdk4
Ppargc1a
Glut4

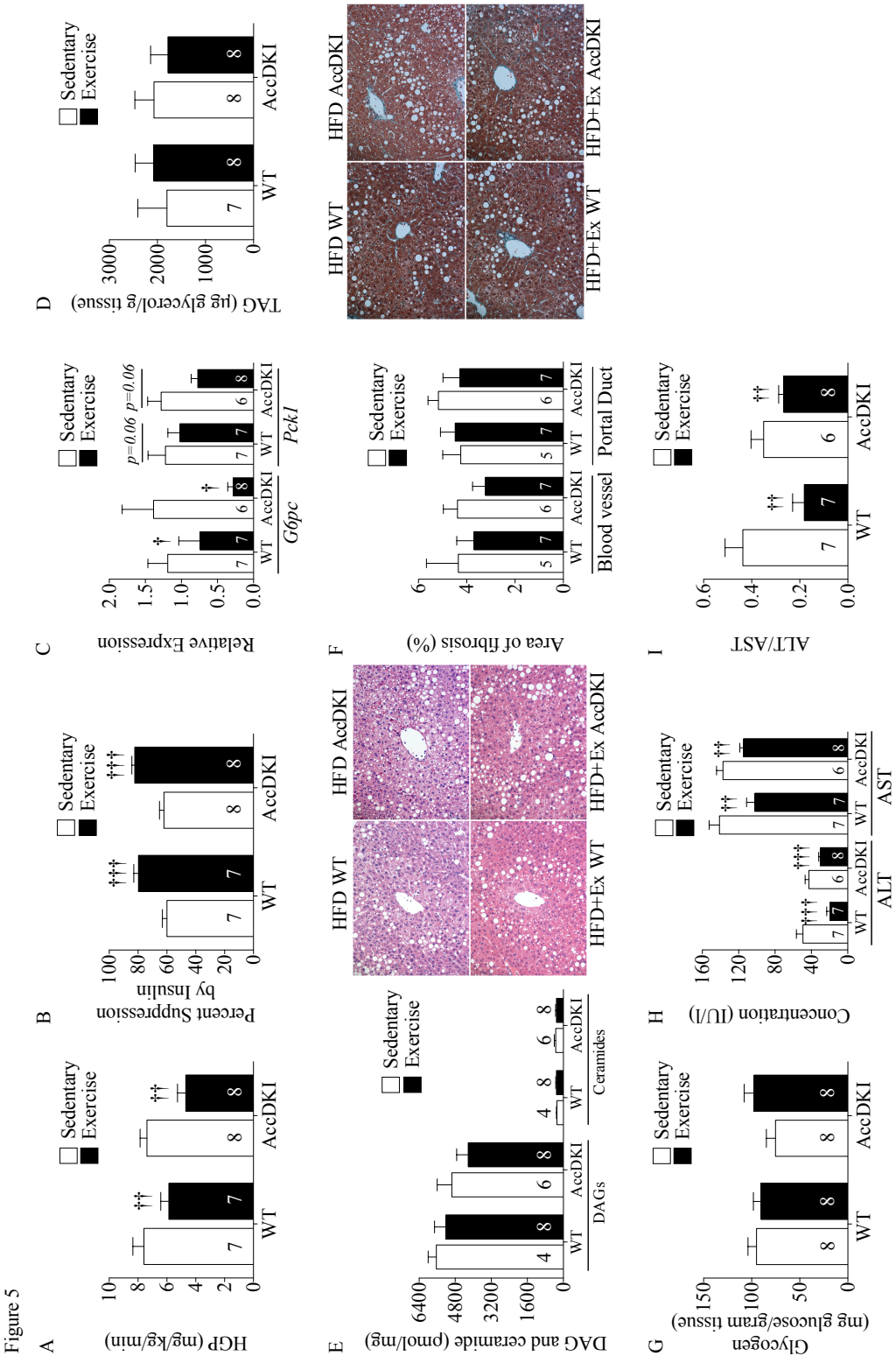

A
HGP (mg/kg/min)
Sedentary
Exercise
WT
AccDKI
B
Percent Suppression by Insulin
C
Relative Expression
Gόpc
Pck1
p=0.06 p=0.06
D
TAG (μg glycerol/g tissue)
E
DAG and ceramide (pmol/mg)
DAGs
Ceramides
F
Area of fibrosis (%)
Blood vessel
Portal Duct
HFD WT
HFD AccDKI
HFD+Ex WT
HFD+Ex AccDKI
G
Glycogen (mg glucose/gram tissue)
H
Concentration (IU/l)
ALT
AST
I
ALT/AST

Figure 5

Table 1: Analysis of tissue weights.

	HFD						HFD+Exercise					
	WT			AccDKI			WT			AccDKI		
	Average	±SEM	N	Average	±SEM	N	Average	±SEM	N	Average	±SEM	N
Liver												
Weight (mg)	1275.3	52.8	6	1162.9	35.1	8	1237.9	79.2	8	1260.6	91.9	8
Weight/gram bw (mg/g bw)	33.4	1.7	6	30.6	1.0	8	32.2	1.3	8	32.1	1.5	8
Epididymal Adipose												
Weight (mg)	2018.1	191.6	6	2375.4	112.7	8	2087.1	74.3	8	2213.0	82.0	8
Weight/gram bw (mg/g bw)	52.7	7.1	6	62.34	2.7	8	55.1	2.7	8	57.0	2.5	8
Heart												
Weight (mg)	129.7	3.4	6	119.7	4.1	8	132.5	4.1	8	133.1	3.1	8
Weight/gram bw (mg/g bw)	3.3	0.1	6	3.2	0.1	8	3.5	0.1	8	3.4	0.1	8

Data are expressed as means ± SEM.

Table 2: Serum cytokine levels.

	HFD						HFD+Exercise					
	WT			AccDKI			WT			AccDKI		
	Average	**±SEM**	**N**	**Average**	**±SEM**	**N**	**Average**	**±SEM**	**N**	**Average**	**±SEM**	**N**
Leptin (ng/mL)	10916.3	798.7	10	9783.7	776.9	7	6466.9***	852.8	9	9570.4	920.7	10
IL-6 (ρg/mL)	9.9	0.6	10	7.9	1.0	6	8.0	0.5	10	10.9	1.1	10
tPAI-1 (ng/mL)	2130.4	307.5	10	2624.3	855.2	7	2045.7	295.2	10	1950.1	263.5	10
Resistin (ng/mL)	4156.0	300	10	4153.8	466.7	8	4080.1	450.6	9	4502.5	483.6	10
TNF-α (ρg/mL)	Not detectable		10	Not detectable		7	Not detectable		9	Not detectable		10
MCP-1 (ρg/mL)	Not detectable		10	Not detectable		7	Not detectable		9	Not detectable		10

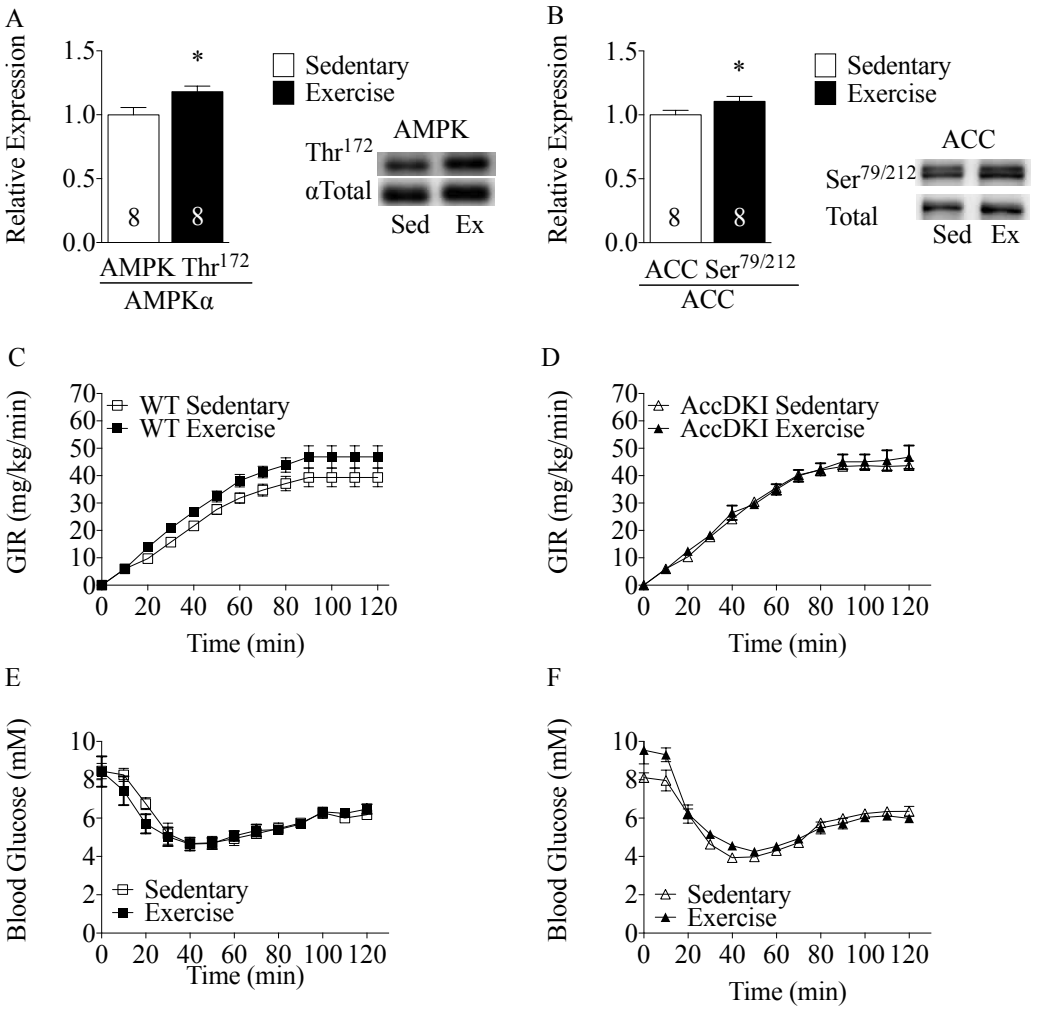
Data are expressed as means ± SEM, $^{***}p<0.001$, for difference between WT sedentary vs WT exercise; $^{§§}p<0.01$, for difference from WT vs AccDKI, as determined by two-way ANOVA and Bonferonni *post hoc* test.

Table 3: Analysis of liver inflammatory markers.

	HFD						HFD+Exercise					
	WT			AccDKI			WT			AccDKI		
	Average	**±SEM**	**N**	**Average**	**±SEM**	**N**	**Average**	**±SEM**	**N**	**Average**	**±SEM**	**N**
Il1b	1.16	0.33	7	0.95	0.15	6	1.13	0.26	7	1.19	0.20	8
Tnfa	1.11	0.22	7	1.56	0.31	6	2.93†††	0.74	7	3.12†††	0.51	8
Erm1	1.03	0.14	7	1.43	0.14	6	2.24†††	0.19	7	2.13†††	0.27	8
Il10	1.13	0.28	7	1.52	0.37	6	1.60	0.33	7	1.61	0.30	8
Nos2	1.08	0.21	7	1.64	0.54	6	0.73	0.29	7	0.94	0.28	8
Arg	1.10	0.24	7	0.94	0.11	6	0.87	0.09	7	0.69	0.10	8
Cd68	1.06	0.18	7	0.97	0.47	6	0.69†	0.07	7	0.76†	0.04	8
Kc	1.26	0.40	7	1.53	0.36	6	0.61†††	0.22	7	0.64†††	0.15	8

Data are expressed as means ± SEM, $^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$, for difference from sedentary vs exercise, as determined by two-way ANOVA and Bonferonni *post hoc* test.

Supplementary Figure 1



CHAPTER FOUR

The AMPK activator R419 improves exercise capacity and skeletal muscle insulin sensitivity in obese mice

Katarina Marcinko, Adam L. Bujak, James J.S. Lally, Rebecca J. Ford, Tammy H. Wong, Brennan Smith BK, Kemp BE, Jenkins Y, Li W, Kinsella TM, Hitoshi Y, Steinberg GR.

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Skeletal muscle AMPK is an important regulator of glucose homeostasis, mitochondrial content and exercise capacity. This manuscript shows that ablation of AMPK in skeletal muscle does not alter high-fat diet-induced insulin resistance in mice. A lack of skeletal muscle AMPK impairs exercise capacity in mice, likely as a result of an inability to achieve exercise-induced skeletal muscle glucose uptake. We also considered the impact of novel AMPK activator R419 in obese mice. We show that R419 improves skeletal muscle glucose uptake to improve whole-body insulin sensitivity and it does so via a skeletal muscle AMPK-independent fashion. Furthermore, R419 improves exercise capacity in obese mice via an AMPK-dependent pathway. These findings suggest that R419 may have therapeutic importance in improving exercise capacity and skeletal muscle insulin sensitivity.

KM, YH and GRS conception and design of research; KM, ALB, JSL, RJF, THW, BKS, YJ, WL, TMK performed experiments; KM, ALB, JSL, THW, BKS analyzed data; KM, YH and GRS interpreted results of experiments; KM and GRS prepared figures; KM, YH and GRS drafted manuscript; KM, BEK, YH, YJ and GRS edited and revised manuscript; KM, ALB, JSL, RJF, THW, BKS, YJ, WL, TMK, BEK, YH and GRS approved final version of manuscript. KM and GRS are responsible for the integrity of the work as a whole.

Brief communication

MOLECULAR METABOLISM

The AMPK activator R419 improves exercise capacity and skeletal muscle insulin sensitivity in obese mice

Katarina Marcinko [1], Adam L. Bujak [1], James S.V. Lally [1], Rebecca J. Ford [1], Tammy H. Wong [1], Brennan K. Smith [1], Bruce E. Kemp [3], Yonchu Jenkins [4], Wei Li [4], Todd M. Kinsella [4], Yasumichi Hitoshi [4], Gregory R. Steinberg [1,2,*]

ABSTRACT

Objective: Skeletal muscle AMP-activated protein kinase (AMPK) is important for regulating glucose homeostasis, mitochondrial content and exercise capacity. R419 is a mitochondrial complex-I inhibitor that has recently been shown to acutely activate AMPK in myotubes. Our main objective was to examine whether R419 treatment improves insulin sensitivity and exercise capacity in obese insulin resistant mice and whether skeletal muscle AMPK was important for mediating potential effects.

Methods: Glucose homeostasis, insulin sensitivity, exercise capacity, and electron transport chain content/activity were examined in wildtype (WT) and AMPK β1β2 muscle-specific null (AMPK-MKO) mice fed a high-fat diet (HFD) with or without R419 supplementation.

Results: There was no change in weight gain, adiposity, glucose tolerance or insulin sensitivity between HFD-fed WT and AMPK-MKO mice. In both HFD-fed WT and AMPK-MKO mice, R419 enhanced insulin tolerance, insulin-stimulated glucose disposal, skeletal muscle 2-deoxyglucose uptake, Akt phosphorylation and glucose transporter 4 (GLUT4) content independently of alterations in body mass. In WT, but not AMPK-MKO mice, R419 improved treadmill running capacity. Treatment with R419 increased muscle electron transport chain content and activity in WT mice; effects which were blunted in AMPK-MKO mice.

Conclusions: Treatment of obese mice with R419 improved skeletal muscle insulin sensitivity through a mechanism that is independent of skeletal muscle AMPK. R419 also increases exercise capacity and improves mitochondrial function in obese WT mice; effects that are diminished in the absence of skeletal muscle AMPK. These findings suggest that R419 may be a promising therapy for improving whole-body glucose homeostasis and exercise capacity.

Keywords Exercise-mimetic; Mitochondrial; Diabetes; Obesity; AMPK; Complex-I; R419

1. INTRODUCTION

The AMP-activated protein kinase (AMPK) is an αβγ heterotrimer that plays an important role in regulating whole-body glucose homeostasis and insulin sensitivity (for review see Ruderman et al., 2013 [1] and Steinberg & Jorgensen 2007 [2]). Over the last decade, many activators of AMPK have been shown to improve insulin sensitivity and glycemic control [3–7] through AMPK dependent [8–10] and independent [11–13] pathways. Of these AMPK activators, metformin, which is a mild complex-I inhibitor, has been extensively studied and found to exert its glucose lowering effects primarily by acting in the liver (for review see Foretz et al., 2014 [14]). This liver predominant effect of metformin is believed to be the result of high expression of the organic cation transporter 1 (OCT1) that is required for metformin uptake and thus limits the effectiveness of metformin to act in other tissues such as skeletal muscle [14]. Similarly, direct activators of AMPK like A769662 [15] and salicylate [16] activate AMPK through the β1 subunit which is prevalent in liver but has low expression in skeletal

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Abbreviations: 2-DG, 2-deoxyglucose; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; AMPK-MKO, skeletal muscle-specific AMPK β1β2 floxed Cre-; AUC, area under the curve; COX, cytochrome c oxidase; CT, computed tomography; EDL, extensor digitorum longus; HFD, high-fat diet (45% kcal fat); GDR, glucose disposal rate; GIR, glucose infusion rate; GLUT4, glucose transporter 4; HGO, hepatic glucose output; OXPHOS, proteins involved in oxidative phosphorylation (electron transport chain); R419, N-(1-(4-cyanobenzyl) piperidin-4-yl)-6-(4-(4-methoxybenzoyl) piperidine-1-carbonyl; *Tbp*, TATA-binding protein; TA, tibialis anterior; WT, wildtype

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muscle [17,18]. While recent studies have identified new direct activators of AMPK that may be effective in muscle [19–21], their role in regulating skeletal muscle insulin sensitivity and muscle mitochondrial content is currently not known. As improvements in skeletal muscle insulin sensitivity are important for restoring glucose homeostasis, there is a need to develop and evaluate new activators of AMPK that may exert positive effects on skeletal muscle metabolism and insulin sensitivity.

In addition to improving insulin sensitivity, AMPK is also vital for controlling muscle mitochondrial content and exercise capacity (for review see Richter & Ruderman 2009 [22] and O'Neill et al., 2013 [23]). For example, mice with undetectable levels of AMPK activity due to genetic deletion of α or β subunits in skeletal muscle have a severely impaired capacity for treadmill running that is associated with alterations in mitochondrial content and/or function [24,25]. In contrast, the activation of AMPK through genetic manipulation or a wide variety of pharmacological agents has been shown to increase treadmill exercise performance in most studies [10,26–29]. These data indicate that activators of AMPK may also be able to increase muscle mitochondrial content and improve exercise performance.

R419 (N-(1-(4-cyanobenzyl)piperidin-4-yl)-6-(4-(4-methoxybenzoyl) piperidine-1-carbonyl)nicotinamide) is a recently described mitochondrial complex I inhibitor that acutely activates AMPK in a variety of cell systems including myotubes and hepatocytes [30]. R419 acutely increases glucose uptake and fatty acid oxidation in cultured myotubes via an AMPK dependent pathway. In contrast, R419 was shown to acutely inhibit glucose production in isolated hepatocytes via an AMPK-independent process. Metabolite tracer analysis of *db/db* mice treated with R419 suggested increased flux through both skeletal muscle glycolytic and fatty acid oxidative pathways and increases in glucose transporter 4 (GLUT4) promoter activity [30]. However, whether chronic R419 treatment improves glucose homeostasis and insulin sensitivity is not currently understood. Since the tissue specific uptake of R419 would not be limited by OCT1, as is the case with metformin, we hypothesized that R419 would improve glucose homeostasis through improvements in skeletal muscle insulin sensitivity and that this effect would require skeletal muscle AMPK. Furthermore, since activation of AMPK has been associated with muscle mitochondrial biogenesis, we also hypothesized that R419 would increase exercise capacity through an AMPK dependent process. We find that in obese mice fed a high-fat diet (HFD), R419 improves glucose tolerance and enhances insulin-stimulated glucose disposal into skeletal muscle in WT and AMPK-MKO mice while also enhancing exercise capacity only in WT controls.

2. MATERIAL AND METHODS

2.1. Mouse experiments

Male AMPK $\beta1\beta2$ floxed muscle creatine kinase (MCK)-Cre- (WT) and AMPK $\beta1\beta2$ floxed MCK-Cre+ (AMPK-MKO) littermates were used in all experiments and have been described previously [25]. The McMaster University (Hamilton, Canada) Animal Ethics Committee approved all experimental protocols. To measure R419-mediated AMPK activation, WT and AMPK-MKO mice were fasted for 12 h and re-fed *ad libitum* with HFD (45 kcal% fat, D12451, Research Diets; New Brunswick, NJ) formulated with R419 at a dose of 100 mg/kg HFD (HFD + R419) or the same HFD without R419. After 3 h of re-feeding, blood glucose was measured by glucometer with a small nick of the tail vein. Mice were then anesthetized and tibialis anterior (TA) muscle was collected, snap frozen and kept at minus 80 °C for later analysis. To examine the chronic effects of R419 treatment, mice were maintained on a 12 h light/dark cycle and fed a HFD starting at 6–8 weeks of age for 12 weeks. After the first 6 weeks of HFD, mice were continued on HFD or given HFD + R419. Mice were allocated to different experiments in order to reduce stress from excessive testing and handling. Food intake was measured over a 72 h timeframe using the Oxymax Lab Animal Monitoring System (Columbus Instruments, OH).

2.2. Exercise capacity test

Mice were acclimated to the treadmill as described previously [17,25,31]. Exercise capacity test was completed once after 5 weeks of treatment. Mice began running at 8 m/min and treadmill speed was increased by 1 m/min every 2 min as previously described [17,25]. At exhaustion, time and speed were recorded and distance traversed calculated. Exhaustion was defined as the point at which instead of running on the treadmill, mice remained on the shockers that serve to encourage running for more than 10 s. Experimenters were unaware of the genotype/treatment of the mice while they were performing the experiment. Mice were quickly removed from the treadmill, blood was collected by facial bleed, and blood glucose recorded. Lactate Assay Kit was used to measure serum lactate, according to manufacturer instructions (Biovision), in the fed basal state and at exhaustion.

2.3. Metabolic studies

After 6 weeks of treatment, computed tomography (CT) was used to assess whole body adiposity and analyzed with the Amira Visage Imaging Software Program, as described previously [10]. Mice were fasted for 12 h in the evening and ~100 μL of blood was collected by facial bleed in the morning. Blood glucose concentrations were recorded by glucometer. Serum analysis of insulin (Millipore) and non-esterified free fatty acids (NEFA) (Wako NEFA-HR) were completed, according to manufacturer instructions. For glucose (GTT, after 5 weeks of treatment) and insulin (ITT, after 6 weeks of treatment) tolerance tests, mice were fasted for 6 h and injected with D-glucose (1 g/kg) and human insulin (1 U/kg, NovoRapid), respectively. Blood glucose was monitored during a 2 h span. Blood glucose was measured by glucometer from a small nick of the tail vein. Hyperinsulinemic-euglycaemic clamps were performed after 12 weeks of the study as previously described [32,33]. The jugular vein was surgically cannulated and if mice had <10% loss in body weight change, they were clamped 5 days later. On the day of the clamp, mice were fasted for 6 h and basal D- [3-^{3}H]-glucose (7.5 μCi/h, 0.12 ml/h in 0.9% saline) was infused for 1 h. This was followed by a constant infusion of insulin (10 mU/kg/min insulin in 0.9% saline) (Novorapid), containing D- [3-^{3}H]-glucose (7.5 μCi/h, 0.12 ml/h in 0.9% saline). Blood glucose was titrated with infusion of 50% dextrose that was gradually increased until euglycaemia was reached, defined as blood glucose between 5.8 and 7.0 mmol/L for at least 30 min [32]. Additionally, glucose uptake in tissues was measured after intravenous injection of 2-[^{14}C]-deoxy-glucose (2-[^{14}C] DG) (10 μCi). Blood samples were taken at 10, 20, and 30 min. Mice were euthanized with an intravenous injection of ketamine-xylazine and sacrificed by exsanguination. Tissues were removed, snap frozen in liquid nitrogen, and stored at minus 80 °C for later analysis [10].

2.4. Analytical techniques

Extensor digitorum longus (EDL), TA and quadriceps muscles were powdered on dry ice and homogenized in cell lysis buffer using a Precellys 24 Homogenizer (Bertin Technologies; Paris, France). Lysates were collected from centrifuged homogenate (13 000 rpm). Tissue-specific glucose uptake (2-[^{14}C] DG-Phosphate) was quantified by scintillation counting in which the unphosphorylated 2-[^{14}C] DG

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fraction was subtracted from the total fraction of homogenate. Values were expressed to blood glucose and 2-[^{14}C] DG infusion in the blood. Additionally, lysates were used to measure cytochrome *c* oxidase (COX) activity, as described previously [34]. Blood was collected for serum biochemistry measurements post-hyperinsulinemic euglycemic clamp, including NEFA and insulin (Iso-insulin ELISA kit (Mercodia)). Steele's equation for steady state conditions was used to determine rates of hepatic glucose output and glucose disposal in the basal and clamped state [35]. Lipid content in quadriceps muscle was measured by Bligh and Dyer procedure [36]. Briefly, lipids were extracted using chloroform and methanol. Lipids from chloroform phase were saponified and glycerol content measured with Glycerol Reagent (Sigma). Glycogen content was measured by incubating powdered tissue in 6N HCl at 80 °C and then neutralized with 6N NaOH. Glucose content was measured using a Glucose Assay (Sigma).

2.5. Western blotting

SDS-PAGE system was used to separate protein extracts. Immunoblotting was performed once proteins were transferred on nitrocellulose or polyvinylidene difluoride membranes. Membranes were first blocked for 1 h at room temperature with 5% BSA in 1xTBST (Tris buffered saline-Tween-20, 25 mM Tris·HCl (pH 7.5), 1 mM NaCl, and 0.1% Tween-20). Membranes were then incubated overnight at 4 °C with primary antibodies (from Cell Signaling, unless indicated) phosphorylated (p) acetyl-CoA carboxylase Ser$^{79/212}$ (1:1000), ACC total (1:1000), pAMPK Thr^{172} (1:1000), AMPK total (1:1000), pAkt Ser^{473} (1:1000), pAkt Thr^{308} (1:1000), Akt (1:1000), GLUT4 (Millipore, 1:1000), OXPHOS (Complex I subunit NDUFB8, Complex II subunit CII-30, Complex III subunit Core 2 CIII-core2, Complex IV subunit I CIV-I, and ATP synthase subunit alpha CV-alpha) (MitoSciences, 1:5000), and GAPDH (Cell Signaling, 1:10 000) in TA and/or quadriceps muscle. Membranes were then washed (3 × 5 min) with TBST and incubated at room temperature for 1 h with corresponding secondary antibody. Membranes were washed (3 × 5 min) with TBST and developed with Clarity™ Western ECL Substrate (Biorad). Densitometry was performed using ImageJ software.

2.6. Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Results were analyzed using a two-way ANOVA with Bonferroni post-hoc tests for group comparisons (unless otherwise denoted), using GraphPad Prism software. Repeated measures ANOVA was used to analyze body mass, GTT, ITT, and GIR. Significance was accepted at $p \leq 0.05$.

3. RESULTS

3.1. R419 acutely activates skeletal muscle AMPK but does not result in hypoglycemia

Treatment with R419 at 100 mg/kg HFD resulted in a plasma concentration of about 24 ng/mL and AUC of 600 ng*hr/mL over 24 h (Figure 1A), which is comparable to previous administration of R419 (5–10 mg/kg body weight) by oral gavage [30]. In WT, but not AMPK-MKO TA muscle, R419 resulted in a tendency for increased AMPK Thr^{172} phosphorylation and significant increases in ACC Ser$^{79/212}$ phosphorylation compared to HFD alone (Figure 1B). It should be noted that ACC Ser$^{79/212}$ phosphorylation is considered a more sensitive measure of cellular AMPK activity as it takes into account the significant role that allosteric and covalent regulation of AMPK has on its activity [37]. R419 treatment did not alter blood glucose in either WT or AMPK-MKO mice relative to HFD alone (Figure 1C).

3.2. A lack of skeletal muscle AMPK or treatment with R419 does not alter weight gain or adiposity in mice fed a HFD

Subsequent experiments were performed in WT and AMPK-MKO mice chronically treated with HFD + R419. A loss of skeletal muscle AMPK did not exacerbate the development of HFD-induced obesity or weight gain over time (Figure 1D). Treatment with HFD + R419 did not result in differences in weight gain (Figure 1D), food intake (Figure 1E) or adiposity (Figure 1F) between groups. There was also no effect of R419 on liver, adipose tissue, soleus, or EDL mass (Table 1). We were specifically interested in the effects of R419 in obesity, therefore, we did not test the effects of the compound in control chow-fed mice but the weight gain and adiposity obtained in these HFD-fed mice were very comparable to our recent publications using the same HFD and time course in which chow control mice were examined [33,38].

3.3. R419 improves insulin sensitivity independent of skeletal muscle AMPK

HFD AMPK-MKO mice had comparable fasting blood glucose and insulin levels compared to WT littermates irrespective of R419 treatment (Figure 2A,B). AMPK-MKO mice had higher fasting serum NEFA (Table 1). Glucose and insulin tolerance and corresponding areas under the curve (AUC) were also similar between AMPK-MKO and WT littermates fed a HFD (Figure 2C,D), indicating that a lack of AMPK does not promote the dysregulation of glucose homeostasis. Of note, the degree of glucose and insulin intolerance achieved in the HFD-fed mice of this study was comparable to many previous studies from our laboratory in which normal chow diet fed controls were also examined in parallel [33,38].

Treatment of mice with R419 did not alter fasting blood glucose (Figure 2A) but it did significantly reduce fasting serum insulin in both WT and AMPK-MKO mice (Figure 2B). Consistent with these findings, R419 tended to improve glucose tolerance and significantly increased whole-body insulin tolerance in both WT and AMPK-MKO mice (Figure 2C,D). These data indicate that R419 improves whole-body insulin sensitivity in obese mice through a pathway independent of skeletal muscle AMPK.

To examine the tissues contributing to these improvements in insulin sensitivity, we conducted hyperinsulinemic-euglycaemic clamps. R419 treatment did not affect basal blood glucose or basal glucose turnover (Supplementary Table 1). Clamped serum glucose and insulin levels were comparable between groups (Supplementary Table 1). However, R419 treatment increased insulin-stimulated glucose infusion rates (GIR) in both WT and AMPK-MKO mice (Figure 2E,F). Consistent with an elevated GIR, the insulin-stimulated glucose disposal rate (GDR) was increased in both WT and AMPK-MKO mice treated with R419 (Figure 2G). Enhanced GDR with R419 treatment was accompanied by elevated 2-[^{14}C]DG uptake at the completion of the clamp into all muscle types of both WT and AMPK-MKO mice (Figure 2H). There were no differences between groups in circulating NEFA (Supplementary Table 1), suggesting that adipose tissue insulin sensitivity was not altered with R419 treatment. During the clamp, R419 treatment had a modest effect on reducing hepatic glucose output (HGO) and tended to increase the percent (%) suppression of HGO in WT mice (Supplementary Table 1).

To investigate the intracellular mechanisms mediating enhanced insulin-stimulated 2-[^{14}C]DG uptake into muscle, we assessed Akt phosphorylation and GLUT4 expression (Figure 2I). Improvements in skeletal muscle insulin-stimulated glucose disposal with R419 treatment were associated with enhanced phosphorylation of Akt Thr^{308} and Ser^{473} (Figure 2J,K). As AMPK may also increase insulin-stimulated glucose uptake by increasing the transcription of GLUT4

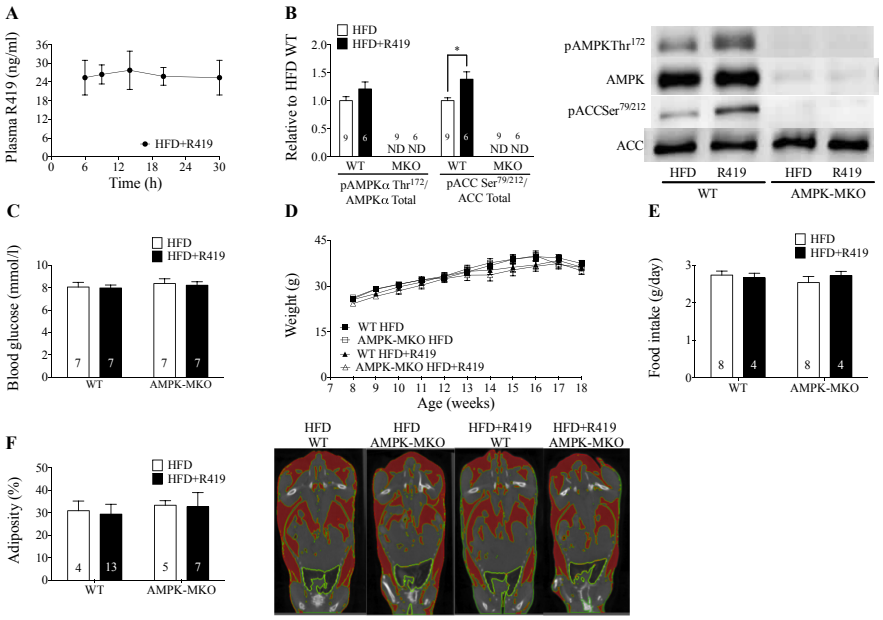

Figure 1: R419 acutely activates skeletal muscle AMPK but does not induce hypoglycemia. A lack of skeletal muscle AMPK or R419 treatment does not alter weight gain or adiposity in mice fed a HFD. (**A**) Plasma concentration of R419 when WT mice were given HFD + R419 at a dose of 100 mg/kg HFD (n = 5). (**B**) AMPK Thr^{172} relative to AMPKα (from separate gels) ($p = 0.16$) and ACC $Ser^{79/212}$ relative to ACC (from separate gels) in TA muscle from WT and AMPK-MKO mice 3 h following HFD or HFD + R419 administration. ND, not detectable. (**C**) Blood glucose concentrations 3 h following HFD or HFD + R419 administration in WT and AMPK-MKO mice. (**D**) Body mass of HFD WT and AMPK-MKO mice treated with or without R419 (two-way repeated measures ANOVA). (**E**) Food intake of HFD WT and AMPK-MKO mice treated with or without R419. (**F**) Measured body percent adiposity with representative CT images. Highlighted red areas represent adipose region. Numbers within bars represent number of mice per group. Data are expressed as means ± SEM, $^{*}p < 0.05$, for difference from HFD vs HFD + R419, as assessed by student's t-test (**B**).

through phosphorylation of histone deacetylases (HDACs) [39], we examined skeletal muscle GLUT4 expression. R419 treatment increased skeletal muscle GLUT4 protein expression in both WT and AMPK-MKO mice (Figure 2L). These data indicate that R419 likely increases glucose disposal in skeletal muscle by increasing both insulin sensitivity and GLUT4 expression via pathways that are independent of skeletal muscle AMPK.

3.4. R419 improves exercise capacity via an AMPK dependent pathway

In agreement with our previous findings in chow-fed mice [25], time and speed at exhaustion were significantly impaired in AMPK-MKO compared to WT mice (Figure 3A,B). Despite obtaining a much lower maximal running speed, AMPK-MKO mice had elevated serum lactates and displayed significantly elevated blood glucose levels, consistent with their inability to stimulate muscle glucose uptake during treadmill running (Table 1), as previously reported [25]. R419 treatment increased time to exhaustion, speed at exhaustion and distance covered in WT but not AMPK-MKO mice (Figure 3A,B,C).

To examine the potential mechanism mediating these effects, we first assessed muscle glycogen and triglycerides and found that R419 did not affect these parameters (Table 1). In HFD-fed mice, mitochondrial complex proteins were reduced in AMPK-MKO mice compared to WT (Figure 3D). R419 increased the protein content of Complex II, III/IV, and V of the respiratory chain in WT mice. These effects of R419 were blunted in AMPK-MKO mice (Figure 3D). There was also a tendency for increased protein expression of Complex I. R419 elevated COX activity in quadriceps (quad) muscle of WT but not AMPK-MKO mice. Collectively, these data suggest that R419 increases mitochondrial biogenesis (Figure 3E).

4. DISCUSSION

R419 has recently been shown to acutely activate AMPK in a variety of cell systems [30] but its effects on whole-body glucose homeostasis, insulin sensitivity and exercise capacity are not known. We demonstrate that chronic R419 treatment lowered fasting insulin, improved glucose tolerance and insulin-stimulated glucose disposal into skeletal muscle, independently of alterations in adiposity in both WT and AMPK-MKO mice. Increases in skeletal muscle 2-DG uptake were associated with elevated Akt phosphorylation and increased GLUT4 expression. In addition, R419 treatment increased exercise capacity

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Table 1 — Analysis of tissue weights, blood glucose, serum lactate, muscle glycogen and triglyceride and serum non-esterified free fatty acids (NEFA).

	AMPK-WT						AMPK-MKO					
	HFD			HFD+R419			HFD			HFD+R419		
	Average	±SEM	N	Average	±SEM	N	Average	±SEM	N	Average	±SEM	N
Liver												
Weight (mg)	1257.9	57.3	14	1192.4	124.4	9	1172.2	60.3	8	1230.0	78.53	8
Weight/gram bw (mg/g bw)	32.5	0.9	14	32.8	2.2	9	34.9	2.5	8	35.6	1.9	8
Epididymal adipose												
Weight (mg)	1299.3	93.9	16	1224.1	104.4	12	1182.8	190.0	8	1173.4	119.9	9
Weight/gram bw (mg/g bw)	34.5	1.7	16	34.5	1.9	12	32.2	4.5	8	33.6	3.1	9
Soleus (mg)	11.4	0.4	13	11.7	0.3	7	11.6	0.5	8	10.9	0.5	7
EDL (mg)	11.0	0.5	9	11.6	0.5	7	10.9	0.9	8	11.2	0.4	7
Glucose (mmol/l)												
Fed	9.1	0.4	13	8.4†	0.3	9	10.3§	0.6	11	9.2†§	0.5	6
Post-Exercise	11.6	0.6	10	9.5	0.5	8	12.5§§	1.2	10	10.3§§	0.4	6
Lactate (mmol/l)												
Fed	6.5	1.0	10	9.3	1.5	7	8.3	1.0	4	8.4	2.2	5
Post-Exercise	8.6	0.8	11	8.1	0.9	7	11.0§	1.3	7	11.1§	0.4	5
Glycogen (mg/g tissue)												
Quadriceps	137.3	6.4	16	141.3	9.8	8	124.9§	3.6	8	118.3§	10.3	8
Triglycerides (μg/g tissue)												
Quadriceps	323.9	29.0	11	371.8	107.5	5	271.1	54.4	6	412.8	57.7	4
NEFA (mmol/l)												
Fasting (12 h)	1.1	0.1	15	1.0	0.1	8	1.6§§§	0.1	6	1.4§§§	0.1	6

Data are expressed as means ± SEM, $^{\dagger}p < 0.05$, for difference from HFD control vs HFD + R419, and $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ for difference from WT vs AMPK-MKO, as determined by two-way ANOVA and Bonferroni *post hoc* test.

and electron transport chain content and activity in WT, but not AMPK-MKO mice. These data indicate that in the context of HFD-induced obesity, R419 may be a promising therapy for improving exercise capacity and glucose homeostasis.

Despite the well-documented role of AMPK activators improving insulin sensitivity, most studies have found that mice having reductions in skeletal muscle AMPK activity fed a control chow [25,34,40] or obesity-promoting HFD diet [40,41] have normal skeletal muscle insulin sensitivity compared to wildtype littermates; although it should be noted that some studies have detected modest reductions in muscle insulin sensitivity [17,42,43]. While previous studies have investigated the effects of an obesity-inducing HFD on a genetic background of lower muscle AMPK activity, it is possible that small amounts of residual AMPK activity may have been sufficient to maintain skeletal muscle insulin sensitivity in these previous reports [40,41]. Thus it was unknown whether AMPK-MKO mice might be more susceptible to HFD-induced obesity and insulin resistance. We found that body mass and adiposity were comparable between genotypes. Insulin tolerance was also unchanged between genotypes as were rates of glucose infusion and glucose disposal during hyperinsulinemic-euglycemic clamps. In contrast to our previous studies in young [25] and aged [34] AMPK-MKO mice which had normal glucose tolerance, we found that there was a tendency for AMPK-MKO mice to have improved glucose tolerance and increased muscle GLUT4 expression when fed HFD. We also found that the large reduction in muscle mitochondrial content we have previously observed in chow-fed AMPK-MKO mice [25] was largely attenuated (so that there were minimal genotype differences) when mice were fed HFD. These data are consistent with findings that HFD-induced mitochondrial biogenesis occurs via a pathway involving calcium/calmodulin-dependent protein kinase [44]. Collectively, these data indicate that a lack of skeletal muscle AMPK does not enhance the development of HFD-induced obesity or insulin resistance and suggest that future studies investigating the induction of compensatory pathways in AMPK-MKO mice in response to HFD are warranted.

Obese mice treated with R419 had lower fasting serum insulin levels, irrespective of genotype. Notable improvements were also observed in glucose and insulin tolerance with R419 treatment. Hyperinsulinemic-euglycaemic clamps revealed that improved insulin sensitivity was mediated by enhanced insulin-stimulated glucose disposal into skeletal muscle and that this effect was independent of skeletal muscle AMPK. In addition to enhancing insulin-stimulated skeletal muscle glucose uptake in a skeletal muscle AMPK independent manner, R419 modestly reduced insulin-stimulated hepatic glucose output; however, it should be noted that the insulin-simulated % suppression of HGO was not significantly different between treatments. Given the modest effect on HGO (and lack of a significant increase in the % suppression), these data suggest, that, in contrast to metformin that primarily elicits its insulin sensitizing effects by acting in the liver [33,45], R419 primarily elicits its glucose lowering/insulin sensitizing effects by enhancing skeletal muscle glucose uptake.

In order to assess the potential mechanisms contributing to the improved insulin-stimulated glucose disposal/2-DG uptake into skeletal muscle following R419 treatment, we assessed activating phosphorylation of Akt and total GLUT4 expression. We found that R419 treated mice had greater Akt phosphorylation and increased GLUT4 expression compared to HFD-fed mice. The overexpression of GLUT4 in skeletal muscle enhances insulin-stimulated glucose uptake and reduces fasting insulin in HFD-fed mice [46]. Enhanced GLUT4 expression may also alter hepatic glucose metabolism/insulin sensitivity, thus explaining the potentially modest changes observed in liver insulin sensitivity [47]. This suggests that increased glucose disposal in skeletal muscle via R419 may be mediated in part through increases in GLUT4 expression via AMPK-independent pathways. These findings are consistent with reports that GLUT4 expression is also increased following exercise via AMPK-independent pathways [48,49]. Future studies investigating the AMPK-independent mechanisms controlling GLUT4 transcription are warranted.

Exercise training is associated with the induction of mitochondrial biogenesis and enhanced exercise performance (for review see Richter

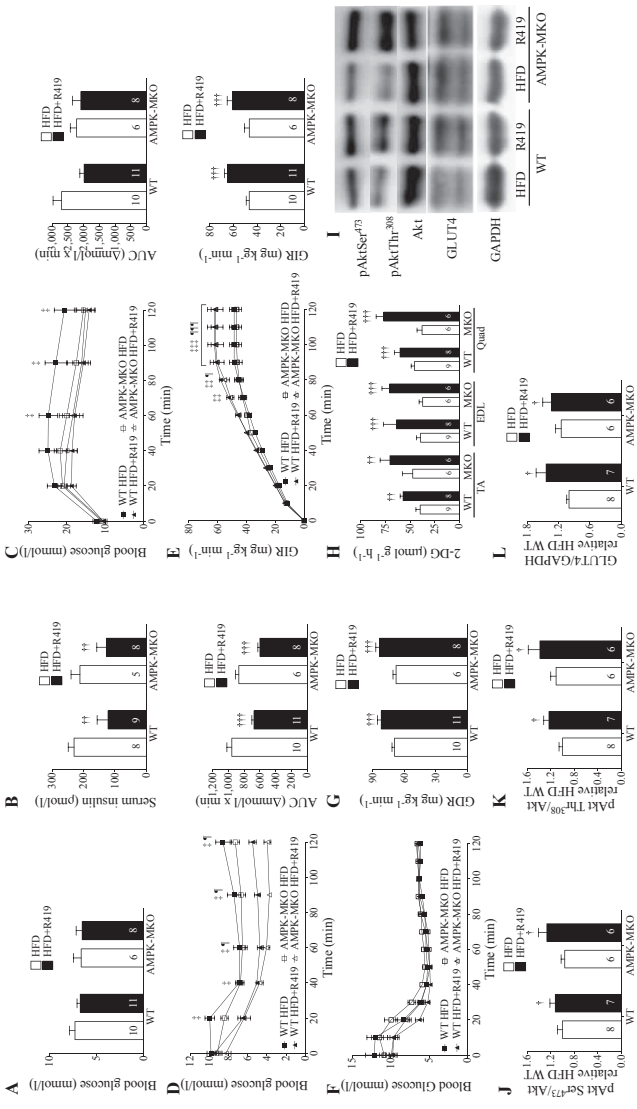

Figure 2: R419 improves insulin sensitivity and insulin-stimulated glucose disposal in skeletal muscle. (**A**) 12 h fasting blood glucose. (**B**) 12 h fasting serum insulin. (**C**) Glucose tolerance test (GTT) and GTT AUC. (**D**) Insulin tolerance test (ITT) and ITT AUC. (**E**) Glucose infusion rate (GIR) curve and clamped GIR. (**F**) Blood glucose concentration curve during clamp. (**G**) Glucose disposal rate (GDR). (**H**) 2-[$^{1-14}$C]DG uptake in TA, EDL, and quadriceps (quad) muscle in WT and AMPK-MKO mice treated with or without R419. (**I**) Representative western blots of pAkt Ser^{473}, pAkt Ser^{308} total Akt, GLUT4, and GAPDH. (**J**) Protein expression of pAkt Ser^{473} relative to Akt (from separate gels). (**K**) Protein expression pAkt Thr^{308} relative to Akt (from separate gels). (**L**) Protein expression of GLUT4 relative to GAPDH. Numbers within bars represent number of mice per group. Data are expressed as means ± SEM. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ for difference from HFD control vs HFD + R419, as determined by two-way ANOVA and Bonferroni *post hoc* test; $^{\ddagger}p < 0.05$, $^{\ddagger\ddagger}p < 0.01$, $^{\ddagger\ddagger\ddagger}p < 0.001$ for difference between HFD WT and HFD + R419 WT, and $^{\P}p < 0.05$, $^{\P\P}p < 0.001$ for difference between HFD AMPK-MKO and HFD + R419 MKO, as determined by two-way repeated measures ANOVA and Bonferroni *post hoc* test (**C,D,E,F**).

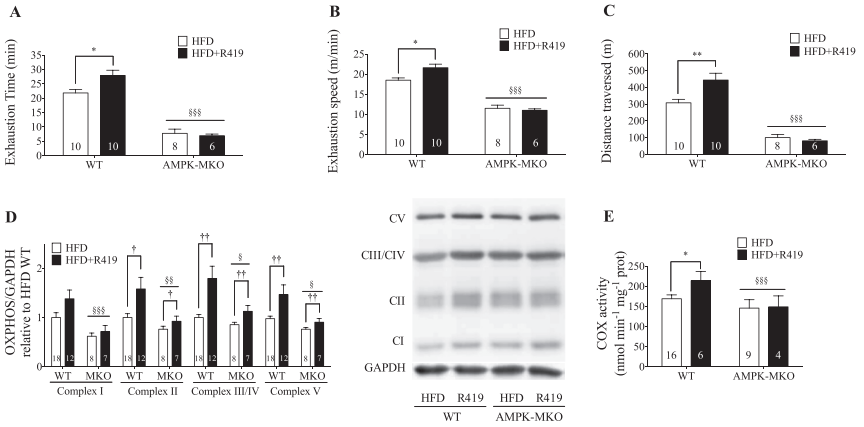

Figure 3: R419 improves exercise capacity via an AMPK dependent pathway involving increased content and activity of the electron transport chain. (**A**) Time to exhaustion. Significant interaction between treatment and genotype ($p = 0.03$). (**B**) Speed at exhaustion. Significant interaction between treatment and genotype ($p = 0.02$). (**C**) Distance traversed. Significant interaction between treatment and genotype ($p = 0.02$). (**D**) OXPHOS complex expression in quadriceps muscle. Representative image of OXPHOS blot from the same membrane. (**E**) COX Activity. Data are expressed as means ± SEM, $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ for difference from HFD control vs HFD + R419; $^{*}p < 0.05$ for difference from HFD control *post hoc* within genotype; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ for difference from WT vs AMPK-MKO, as determined by two-way ANOVA and Bonferroni *post hoc* test.

& Ruderman 2009 [22] and O'Neill et al., 2011 [23]). R419 improved treadmill running capacity in WT mice by over 30%, while having no effect in AMPK-MKO mice. This increase in treadmill running capacity is comparable to other studies in mice with endurance exercise training [50,51]. A limitation of our study was that we defined fatigue/exhaustion as the point at which instead of running on the treadmill, mice remained on the shockers, which serve to encourage running, for more than 10 s. Future studies measuring biochemical measures of exhaustion ([52], e.g. muscle and liver glycogen, lactate) with and without R419 and/or muscle AMPK deficiency will be important to establish genuine fatigue and to confirm the effects were not due to changes in motivation or altered sensitivity to the electrical shocking system.

A feature of studies in which AMPK has been activated using either genetic gain of function [53] or pharmacological agents such as AICAR [54] is that muscle glycogen contents are elevated; which could be a primary factor contributing to the enhanced exercise performance [42]. With R419 treatment, there was no change in muscle glycogen content. Instead, improvements in treadmill running capacity in WT mice were associated with increased protein expression of subunits of the electron transport chain and COX activity. These observations that R419, a complex-I inhibitor, can enhance mitochondrial content and function are consistent with previous findings indicating that siRNA mediated complex-1 inhibition in C. elegans induces mitohormesis (an increase in mitochondrial biogenesis and efficiency) [55]. Interestingly, these effects were blunted in AMPK-MKO mice suggesting that R419 primarily elicits its effects on mitochondrial function through a pathway involving AMPK. Future studies investigating the downstream substrates mediating R419 effects on mitochondrial function are warranted.

In summary, chronic treatment with R419 leads to substantial improvements in glucose homeostasis, effects that are primarily mediated through enhanced skeletal muscle insulin sensitivity and are independent of skeletal muscle AMPK. In addition, chronic treatment of obese mice with R419 elicits improvements in exercise capacity and skeletal muscle electron transport chain content/activity in WT mice, effects which are blunted in the absence of AMPK. These data indicate that R419 mimics many of the effects of chronic exercise training in skeletal muscle and suggest that R419 may be of therapeutic importance for improving exercise capacity and skeletal muscle insulin sensitivity in obesity.

AUTHOR CONTRIBUTIONS

KM, YH and GRS conception and design of research; KM, ALB, JSL, RJF, THW, BKS, YJ, WL, TMK performed experiments; KM, ALB, JSL, THW, BKS analyzed data; KM, YH and GRS interpreted results of experiments; KM and GRS prepared figures; KM, YH and GRS drafted manuscript; KM, BEK, YH, YJ and GRS edited and revised manuscript; KM, ALB, JSL, RJF, THW, BKS, YJ, WL, TMK, BEK, YH and GRS approved final version of manuscript. KM and GRS are responsible for the integrity of the work as a whole.

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CONFLICT OF INTEREST

YJ, WL, TMK and YH are employees of Rigel Pharmaceuticals, Inc. No other potential conflicts of interest relevant to this article were reported.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.06.002

REFERENCES

[1] Ruderman, N.B., Carling, D., Prentki, M., Cacicedo, J.M., 2013. AMPK, insulin resistance, and the metabolic syndrome. The Journal of Clinical Investigation 123(7):2764–2772. http://dx.doi.org/10.1172/JCI67227.

[2] Steinberg, G.R., Jorgensen, S.B., 2007. The AMP-activated protein kinase: role in regulation of skeletal muscle metabolism and insulin sensitivity. Mini-Reviews in Medicinal Chemistry 7(5):519–526.

[3] Al-Khalili, L., Forsgren, M., Kannisto, K., Zierath, J.R., Lönnqvist, F., Krook, A., 2005. Enhanced insulin-stimulated glycogen synthesis in response to insulin, metformin or rosiglitazone is associated with increased mRNA expression of GLUT4 and peroxisomal proliferator activator receptor gamma co-activator 1. Diabetologia 48(6):1173–1179. http://dx.doi.org/10.1007/s00125-005-1741-3.

[4] Chen, Y.-C., Lee, S.-D., Ho, L.-T., Kuo, C.-H., 2011. The effects of Altitude training on the AMPK-related glucose transport pathway in the red skeletal muscle of both Lean and obese Zucker rats. High Altitude Medicine & Biology 12(4):371–378. http://dx.doi.org/10.1089/ham.2010.1088.

[5] Gomes, A.P., Duarte, F.V., Nunes, P., Hubbard, B.P., Teodoro, J.S., Varela, A.T., et al., 2012. Berberine protects against high fat diet-induced dysfunction in muscle mitochondria by inducing SIRT1-dependent mitochondrial biogenesis. Biochimica et Biophysica Acta – Molecular Basis of Disease 1822(2):185–195. http://dx.doi.org/10.1016/j.bbadis.2011.10.008.

[6] Iglesias, M.A., Ye, J.M., Frangioudakis, G., Saha, A.K., Tomas, E., Ruderman, N.B., et al., 2002. AICAR administration causes an apparent enhancement of muscle and liver insulin action in insulin-resistant high-fat-fed rats. Diabetes 51(10):2886–2894. http://dx.doi.org/10.2337/diabetes.52.1.223.

[7] Kumar, N., Dey, C.S., 2002. Metformin enhances insulin signalling in insulin-dependent and-independent pathways in insulin resistant muscle cells. British Journal of Pharmacology 137(3):329–336. http://dx.doi.org/10.1038/sj.bjp.0704878.

[8] Kjøbsted, R., Treebak, J.T., Fentz, J., Lantier, L., Viollet, B., Birk, J.B., et al., 2015. Prior AICAR stimulation increases insulin sensitivity in mouse skeletal muscle in an AMPK-dependent manner. Diabetes 64(6):2042–2055.

[9] Kristensen, J.M., Treebak, J.T., Schjerling, P., Goodyear, L., Wojtaszewski, J.F.P., 2014. Two weeks of metformin treatment induces AMPK-dependent enhancement of insulin-stimulated glucose uptake in mouse soleus muscle. American Journal of Physiology. Endocrinology and Metabolism 306(10):E1099–E1109. http://dx.doi.org/10.1152/ajpendo.00417.2013.

[10] Watt, M.J., Dzamko, N., Thomas, W.G., Rose-John, S., Ernst, M., Carling, D., et al., 2006. CNTF reverses obesity-induced insulin resistance by activating skeletal muscle AMPK. Nature Medicine 12(5):541–548. http://dx.doi.org/10.1038/nm1383.

[11] Yang, Z., Wang, X., He, Y., Qi, L., Yu, L., Xue, B., et al., 2012. The full capacity of AICAR to reduce obesity-induced inflammation and insulin resistance requires Myeloid SIRT1. PLoS One 7(11). http://dx.doi.org/10.1371/journal.pone.0049935.

[12] Turban, S., Stretton, C., Drouin, O., Green, C.J., Watson, M.L., Gray, A., et al., 2012. Defining the contribution of AMP-activated protein kinase (AMPK) and protein kinase C (PKC) in regulation of glucose uptake by metformin in skeletal muscle cells. Journal of Biological Chemistry 287(24):20088–20099. http://dx.doi.org/10.1074/jbc.M111.330746.

[13] Lee-Young, R.S., Bonner, J.S., Mayes, W.H., Iwueke, I., Barrick, B.A., Hasenour, C.M., et al., 2013. AMP-activated protein kinase (AMPK)α2 plays a role in determining the cellular fate of glucose in insulin-resistant mouse skeletal muscle. Diabetologia 56(3):608–617. http://dx.doi.org/10.1007/s00125-012-2787-7.

[14] Foretz, M., Guigas, B., Bertrand, L., Pollak, M., Viollet, B., 2014. Metformin: from mechanisms of action to therapies. Cell Metabolism 20(6):953–966.

[15] Scott, J.W., van Denderen, B.J.W., Jorgensen, S.B., Honeyman, J.E., Steinberg, G.R., Oakhill, J.S., et al., 2008. Thienopyridone drugs are selective activators of AMP-activated protein kinase β1-containing complexes. Chemistry and Biology 15(11):1220–1230. http://dx.doi.org/10.1016/j.chembiol.2008.10.005.

[16] Hawley, S.A., Fullerton, M.D., Ross, F.A., Schertzer, J.D., Chevtzoff, C., Walker, K.J., et al., 2012. The Ancient drug salicylate directly activates AMP-activated protein kinase. Science 336(6083):918–922. http://dx.doi.org/10.1126/science.1215327.

[17] Steinberg, G.R., O'Neill, H.M., Dzamko, N.L., Galic, S., Naim, T., Koopman, R., et al., 2010. Whole body deletion of AMP-activated protein kinase {beta}2 reduces muscle AMPK activity and exercise capacity. The Journal of Biological Chemistry 285(48):37198–37209. http://dx.doi.org/10.1074/jbc.M110.102434.

[18] Wojtaszewski, J.F.P., Birk, J.B., Frøsig, C., Holten, M., Pilegaard, H., Dela, F., 2005. 5′ AMP activated protein kinase expression in human skeletal muscle: effects of strength training and type 2 diabetes. Journal of Physiology 564(2):563–573. http://dx.doi.org/10.1113/jphysiol.2005.082669.

[19] Hunter, R.W., Foretz, M., Bultot, L., Fullerton, M.D., Deak, M., Ross, F.A., et al., 2014. Mechanism of action of compound-13: an α1-selective small molecule activator of AMPK. Chemistry and Biology 21(7):866–879. http://dx.doi.org/10.1016/j.chembiol.2014.05.014.

[20] Jensen, T.E., Ross, F.A., Kleinert, M., Sylow, L., Knudsen, J.R., Gowans, G.J., et al., 2015. PT-1 selectively activates AMPK-γ1 complexes in mouse skeletal muscle, but activates all three γ subunit complexes in cultured human cells by inhibiting the respiratory chain. Biochemical Journal 467(3):461–472.

[21] Lai, Y.-C., Kviklyte, S., Vertommen, D., Lantier, L., Foretz, M., Viollet, B., et al., 2014. A small-molecule benzimidazole derivative that potently activates AMPK to increase glucose transport in skeletal muscle: comparison with effects of contraction and other AMPK activators. The Biochemical Journal 460(3):363–375. http://dx.doi.org/10.1042/BJ20131673.

[22] Richter, E.A., Ruderman, N.B., 2009. AMPK and the biochemistry of exercise: implications for human health and disease. The Biochemical Journal 418(2):261–275. http://dx.doi.org/10.1042/BJ20082055.

[23] O'Neill, H.M., Holloway, G.P., Steinberg, G.R., 2013. AMPK regulation of fatty acid metabolism and mitochondrial biogenesis: implications for obesity. Molecular and Cellular Endocrinology 366(2):135–151. http://dx.doi.org/10.1016/j.mce.2012.06.019.

[24] Lantier, L., Fentz, J., Mounier, R., Leclerc, J., Treebak, J.T., Pehmøller, C., et al., 2014. AMPK controls exercise endurance, mitochondrial oxidative capacity, and skeletal muscle integrity. FASEB Journal 28(7):3211–3224. http://dx.doi.org/10.1096/fj.14-250449.

[25] O'Neill, H.M., Maarbjerg, S.J., Crane, J.D., Jeppesen, J., Jorgensen, S.B., Schertzer, J.D., et al., 2011. AMP-activated protein kinase (AMPK) 1 2 muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. Proceedings of the National Academy of Sciences 108(38):16092–16097. http://dx.doi.org/10.1073/pnas.1105062108.

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[26] Barnes, B.R., Glund, S., Long, Y.C., Hjälm, G., Andersson, L., Zierath, J.R., 2005. 5′-AMP-activated protein kinase regulates skeletal muscle glycogen content and ergogenics. FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology 19(7):773–779. http://dx.doi.org/10.1096/fj.04-3221com.

[27] Rockl, K.S.C., Hirshman, M.F., Brandauer, J., Fujii, N., Witters, L.A., Goodyear, L.J., 2007. Skeletal muscle adaptation to exercise training - AMP-activated protein kinase mediates muscle fiber type shift. Diabetes 56(8): 2062–2069. http://dx.doi.org/10.2337/db07-0255.

[28] Winder, W.W., Holmes, B.F., Rubink, D.S., Jensen, E.B., Chen, M., Holloszy, J.O., 2000. Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. Journal of Applied Physiology (Bethesda, Md.: 1985) 88(6):2219–2226.

[29] Zong, H., Ren, J.M., Young, L.H., Pypaert, M., Mu, J., Birnbaum, M.J., et al., 2002. AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. Proceedings of the National Academy of Sciences of the United States of America 99(25):15983–15987. http://dx.doi.org/10.1073/pnas.252625599.

[30] Jenkins, Y., Sun, T.Q., Markovtsov, V., Foretz, M., Li, W., Nguyen, H., et al., 2013. AMPK activation through mitochondrial regulation results in increased substrate oxidation and improved metabolic parameters in models of diabetes. PLoS One 8(12). http://dx.doi.org/10.1371/journal.pone.0081870.

[31] Dzamko, N., Schertzer, J.D., Ryall, J.G., Steel, R., Macaulay, S.L., Wee, S., et al., 2008. AMPK-independent pathways regulate skeletal muscle fatty acid oxidation. The Journal of Physiology 586(Pt 23):5819–5831. http://dx.doi.org/10.1113/jphysiol.2008.159814.

[32] Galic, S., Fullerton, M.D., Schertzer, J.D., Sikkema, S., Marcinko, K., Walkley, C.R., et al., 2011. Hematopoietic AMPK1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. Journal of Clinical Investigation 121(12):4903–4915. http://dx.doi.org/10.1172/JCI58577.

[33] Fullerton, M.D., Galic, S., Marcinko, K., Sikkema, S., Pulinilkunnil, T., Chen, Z.-P., et al., 2013. Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. Nature Medicine 19(12):1649–1654. http://dx.doi.org/10.1038/nm.3372.

[34] Bujak, A.L., Blümer, R.M.E., Marcinko, K., Fullerton, M.D., Kemp, B.E., Steinberg, G.R., 2014. Reduced skeletal muscle AMPK and mitochondrial markers do not promote age-induced insulin resistance. Journal of Applied Physiology (Bethesda, Md.: 1985) 117(2):171–179. http://dx.doi.org/10.1152/japplphysiol.01101.2013 (May).

[35] Steele, R., 1959. Influences of glucose loading and of injected insulin on hepatic glucose output. Annals of the New York Academy of Sciences 82: 420–430.

[36] Bligh, E.G., Dyer, W.J., 1959. A rapid Method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37(8):911–917. http://dx.doi.org/10.1139/o59-099.

[37] Gowans, G.J., Hawley, S.A., Ross, F.A., Hardie, D.G., 2013. AMP is a true physiological regulator of amp-activated protein kinase by both allosteric activation and enhancing net phosphorylation. Cell Metabolism 18(4):556–566. http://dx.doi.org/10.1016/j.cmet.2013.08.019.

[38] Jørgensen, S.B., Nielsen, J.N., Birk, J.B., Olsen, G.S., Viollet, B., Andreelli, F., et al., 2004. The α2-5′ AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. Diabetes 53(12):3074–3081. http://dx.doi.org/10.2337/diabetes.53.12.3074.

[39] McGee, S.L., Van Denderen, B.J.W., Howlett, K.F., Mollica, J., Schertzer, J.D., Kemp, B.E., et al., 2008. AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. Diabetes 57(4):860–867. http://dx.doi.org/10.2337/db07-0843.

[40] Beck Jørgensen, S., O'Neill, H.M., Hewitt, K., Kemp, B.E., Steinberg, G.R., 2009. Reduced AMP-activated protein kinase activity in mouse skeletal muscle does not exacerbate the development of insulin resistance with obesity. Diabetologia 52(11):2395–2404. http://dx.doi.org/10.1007/s00125-009-1483-8.

[41] Frøsig, C., Jensen, T.E., Jeppesen, J., Pehmøller, C., Treebak, J.T., Maarbjerg, S.J., et al., 2013. AMPK and insulin action–responses to ageing and high fat diet. PloS One 8(5):e62338. http://dx.doi.org/10.1371/journal.pone.0062338.

[42] Goodyear, L.J., 2008. The exercise pill–too good to be true? The New England Journal of Medicine 359(17):1842–1844 http://dx.doi.org/10.1056/NEJMcibr0806723.

[43] Viollet, B., Andreelli, F., Jørgensen, S.B., Perrin, C., Geloen, A., Flamez, D., et al., 2003. The AMP-activated protein kinase alpha2 catalytic subunit controls whole-body insulin sensitivity. The Journal of Clinical Investigation 111(1): 91–98. http://dx.doi.org/10.1172/JCI16567.

[44] Jain, S.S., Paglialunga, S., Vigna, C., Ludzki, A., Herbst, E. a, Lally, J.S., et al., 2014. High-fat diet-induced mitochondrial biogenesis is regulated by mitochondrial derived reactive oxygen species activation of CaMKII. Diabetes 63(6): 1–15. http://dx.doi.org/10.2337/db13-0816.

[45] Shu, Y., Sheardown, S.A., Brown, C., Owen, R.P., Zhang, S., Castro, R.A., et al., 2007. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. The Journal of Clinical Investigation 117(5): 1422–1431. http://dx.doi.org/10.1172/JCI30558.

[46] Tsao, T.S., Burcelin, R., Katz, E.B., Huang, L., Charron, M.J., 1996. Enhanced insulin action due to targeted GLUT4 overexpression exclusively in muscle. Diabetes 45(1):28–36. http://dx.doi.org/10.2337/diabetes.45.1.28.

[47] Ranalletta, M., Du, X.Q., Seki, Y., Glenn, A.S., Kruse, M., Fiallo, A., et al., 2007. Hepatic response to restoration of GLUT4 in skeletal muscle of GLUT4 null mice. American Journal of Physiology. Endocrinology and Metabolism 293(5): E1178–E1187. http://dx.doi.org/10.1152/ajpendo.00628.2006.

[48] Holmes, B.F., Lang, D.B., Birnbaum, M.J., Mu, J., Dohm, G.L., 2004. AMP kinase is not required for the GLUT4 response to exercise and denervation in skeletal muscle. American Journal of Physiology. Endocrinology and Metabolism 287(4):E739–E743. http://dx.doi.org/10.1152/ajpendo.00080.2004.

[49] Murgia, M., Jensen, T.E., Cusinato, M., Garcia, M., Richter, E.A., Schiaffino, S., 2009. Multiple signalling pathways redundantly control glucose transporter GLUT4 gene transcription in skeletal muscle. The Journal of Physiology 587(Pt 17):4319–4327. http://dx.doi.org/10.1113/jphysiol.2009.174888.

[50] Jordy, A.B., Kraakman, M.J., Gardner, T., Estevez, E., Kammoun, H.L., Weir, J.M., et al., 2015. Analysis of the liver lipidome reveals insights into the protective effect of exercise on high-fat diet-induced hepatosteatosis in mice. American Journal of Physiology – Endocrinology and Metabolism 308(9): E778–E791.

[51] Lund, J., Hafstad, A.D., Boardman, N.T., Rossvoll, L., Rolim, N.P., Ahmed, M.S., et al., 2015. Exercise training promotes cardioprotection through oxygen-sparing action in high fat-fed mice. American Journal of Physiology – Heart and Circulatory Physiology 308(8):H823–H829.

[52] Booth, F.W., Laye, M.J., Spangenburg, E.E., 2010. Gold standards for scientists who are conducting animal-based exercise studies. Journal of Applied Physiology 108:219–221.

[53] Barré, L., Richardson, C., Hirshman, M.F., Brozinick, J., Fiering, S., Kemp, B.E., et al., 2007. Genetic model for the chronic activation of skeletal muscle AMP-activated protein kinase leads to glycogen accumulation. American Journal of Physiology. Endocrinology and Metabolism 292(3):E802–E811. http://dx.doi.org/10.1152/ajpendo.00369.2006.

[54] Aschenbach, W.G., Hirshman, M.F., Fujii, N., Sakamoto, K., Howlett, K.F., Goodyear, L.J., 2002. Effect of AICAR treatment on glycogen metabolism in skeletal muscle. Diabetes 51(3):567–573. http://dx.doi.org/10.2337/diabetes.51.3.567.

[55] Owusu-Ansah, E., Song, W., Perrimon, N., 2013. XMuscle mitohormesis promotes longevity via systemic repression of insulin signaling. Cell 155(3). http://dx.doi.org/10.1016/j.cell.2013.09.021.

Supplementary Table 1: Hyperinsulinemic-euglycemic clamp serum analytes and parameters.

	AMPK-WT						AMPK-MKO					
	HFD			HFD+R419			HFD			HFD+R419		
	Average	±SEM	N	Average	±SEM	N	Average	±SEM	N	Average	±SEM	N
Basal State												
Blood Glucose (mmol/l)	11.7	0.8	10	10.3	0.7	11	10.2	0.4	6	10.5	0.8	8
HGP (mg kg^{-1} min^{-1})	35.4	2.6	10	32.4	1.8	11	28.6	1.7	6	31.2	3.0	8
Clamped State												
HGP (mg kg^{-1} min^{-1})	22.8	2.1	10	14.4‡	2.4	11	21.3	1.9	6	23.4	1.8	8
% Suppression (%)	32.1	7.8	10	51.3	8.4	11	24.2	8.2§§	6	20.0§§	6.6	8
Blood Glucose (mmol/l)	6.4	0.1	10	6.4	0.1	11	6.3	0.1	6	6.3	0.2	8
Steady State Insulin (ρmol/l)	268.0	39.2	8	376.8	118.6	8	378.8	85.1	5	318.8	67.5	4
NEFA (mmol/l)	0.4	0.1	10	0.6	0.1	9	0.6	0.0	6	0.6	0.2	6

CHAPTER FIVE

DISCUSSION

5.1 Introduction

This compilation of studies examined the insulin sensitizing effects of AMPK activation in obese mice. Specifically, it described the effects of AMPK activation by metformin, exercise training, and R419 in mice fed a high-fat diet. In Chapter 2 we show that metformin reduces liver lipid content by increasing AMPK phosphorylation of ACC1 Ser79 and ACC2 Ser212, which results in improvements in hepatic insulin sensitivity. In Chapter 3 we show that exercise-induced improvements in insulin sensitivity occur independent of these ACC phosphorylation sites and independent of lipid content in the liver. Finally, in Chapter 4 we show that R419 improves skeletal muscle insulin sensitivity independent of AMPK and skeletal muscle lipid content but improves exercise capacity in obese mice via a skeletal muscle AMPK-dependent pathway.

5.2 AMPK, metformin, and liver insulin sensitivity

Chapter 2 of this thesis examined chronic effects of metformin in an obese setting using a clinically relevant dose of metformin (50 mg/kg daily for 6 weeks which has been shown to elicit a 29 μM plasma concentration of metformin [476]). We demonstrated that chronic metformin treatment alleviates hepatic insulin resistance via AMPK phosphorylation of ACC1 at Ser79 and ACC2 at

Ser212. Improvements in hepatic insulin sensitivity with metformin in WT mice were associated with reductions in liver *de novo* lipogenesis and liver lipid content (DAG), effects not observed in AccDKI mice. These data suggest that chronic metformin treatment improves insulin sensitivity through a mechanism requiring AMPK phosphorylation of ACC and reductions in liver lipid content. While some groups have shown an important role for AMPK in regulating liver hepatic glucose output [173,431,477], others have not [441,442] and these studies will be discussed in the preceding paragraphs.

Foretz et al. (2010) observed that metformin (250 μM and 1 mM) inhibited gluconeogenesis in AMPK α1α2 KO hepatocytes and lowered blood glucose levels in mice deficient in AMPK after an acute administration of metformin (at doses 150 and 300 mg/kg by oral gavage). Rather than lowering gluconeogenesis through AMPK activation, metformin was proposed to inhibit gluconeogenesis via changes in hepatic energy state which increases AMP levels and activates PKA [441]. Miller et al. (2013) also demonstrated that phenformin (100 μM and 1 mM in hepatocytes), a biguanide like metformin, reduces gluconeogenesis by inhibiting glucagon signaling through a cAMP-PKA pathway [442]. However, it should be noted that phenformin is a stronger complex I inhibitor and has a 20 times greater likelihood of inducing lactic acidosis in humans [478]. In a study examining acute and chronic effects of metformin, Madiraju et al. (2014) showed that a clinically relevant dose of metformin (50 mg/kg-the same dose used in our study) inhibited mitochondrial glycerol 3-

phosphate dehydrogenase and resulted in elevated NADH levels [479]. Interestingly, AMPK phosphorylates and inhibits glycerol 3-phosphate dehydrogenase in yeast [480], which reduces glucose production from glycerol and lactate [479], suggesting that this study may support a role for AMPK in mediating the effects of metformin on hepatic gluconeogenesis.

Clearly scientists are divided in their understanding of how metformin is driving improvements in insulin sensitivity. A concern regarding the studies listed above is the major inconsistencies in the dose used to examine the mechanism by which metformin inhibits hepatic gluconeogenesis. After clinical administration of metformin [481], concentrations of metformin in the hepatic vein and circulation have been shown to be 40-70 μM [476] and 10-40 μM [482,483], respectively. Interestingly, most low metformin concentrations have been shown to have AMPK-dependent effects on gluconeogenesis [173,477], while higher doses appear to mostly have AMPK-independent effects on gluconeogenesis [441,442]. Studies indicating AMPK-independent effects of metformin used concentrations that were 10-100 times higher than physiological levels. In addition, the low (<50 μM) concentrations of metformin in these studies did not change cAMP levels and AMP/ATP ratio but still inhibited glucose production [441,442]. Interestingly, low dose metformin (<100 μM), but not high dose metformin (>500 μM), has also been shown to stabilize the AMPK heterotrimer [484]. This speaks to the need for future animal studies to use physiologically relevant doses of metformin to identify mechanisms of action.

In addition to variability in the dose of metformin used, most studies have focused their attention on the acute effects of metformin and examinations of the mechanism for the chronic effects of metformin in obese insulin resistant mice have not been widely studied. Genome-wide analysis of patients with type 2 diabetes taking metformin has been associated with increased AMPK subunit genes [485] and chronic treatment with metformin increases AMPK Thr172 phosphorylation without inhibiting mitochondrial complex I activity and/or ATP content in the liver of humans and mice [486,487]. Therefore, future studies examining the effects of chronic metformin treatment on hepatic glucose production within the physiological range *in vivo* are necessary.

Recently, Duca et al. (2015) proposed that duodenal AMPK has an important role in regulating hepatic glucose production [488]. Ablation of duodenal AMPK by adenovirus impeded hepatic glucose production reduction and glucose lowering effects of metformin (administered by duodenal infusion at a 200 mg/kg dose). The suggested mechanism of action involves AMPK activation of incretin hormone glucagon like peptide-1 receptor (GLP-1r) in the duodenum. Metformin has previously been shown to stimulate GLP-1 secretion [489], which has been shown to regulate the gut-brain-liver axis maintaining hepatic glucose production [490]. GLP-1r also increases PKA signaling [491]. Using a gut-brain-liver axis, duodenal AMPK activation of GLP-1r and PKA by metformin was proposed to send a neural circuit to the brain which then sends a signal to decrease hepatic glucose production [488]. It should be noted, however,

that this study examined only acute effects of metformin on hepatic glucose production. In addition, metformin (75 mg/kg by oral gavage) has previously been shown to improve glucose tolerance independent of incretin hormones [489]. Therefore, examination of chronic effects of metformin treatment on duodenal AMPK and hepatic glucose production in rodents fed a high-fat diet is warranted. It would also be important to look at AMPK and ACC phosphorylation within the liver after an acute bolus of metformin in the duodenum.

Therefore, to better elucidate the insulin sensitization mechanisms of metformin, future studies should focus on long term dietary supplementation at clinical doses starting on a background of insulin resistance.

5.3 AMPK, exercise, and liver insulin sensitivity

It is of interest that metformin and exercise – two AMPK activators – use different mechanisms to improve hepatic insulin sensitivity. Metformin-induced improvements in hepatic insulin sensitivity were shown to depend on the ability of AMPK to phosphorylate ACC signaling and reduce liver lipid content. In contrast, exercise-induced improvements in hepatic insulin sensitivity occur independent of ACC and liver lipid content. It should be noted that many previous studies examining the effects of exercise on insulin sensitivity were complicated by weight loss [415,416,420–424]. Our exercise training protocol was unique since we show that improvements in hepatic insulin sensitivity can occur without weight loss and without changes in liver lipid content. The next section will

discuss a few different targets that we think should be examined by future studies to assess the mechanism(s) by which exercise training improves insulin sensitivity.

Overexpression of hepatic MCD has been shown to improve muscle, liver, and whole-body insulin sensitivity and reduce liver triglyceride content by lowering free-fatty acid levels [492]. We measured protein expression of liver MCD following the hyperinsulinemic-euglycemic clamp and to our surprise, found that MCD protein expression was reduced in the liver of WT and AccDKI mice that were exercise trained compared to those that were sedentary (Figure 5.1). These data further support the idea that exercise training-induced improvements in hepatic insulin sensitivity are not driven by alterations in malonyl-CoA levels and suggest an alternative pathway is driving the observed improvements.

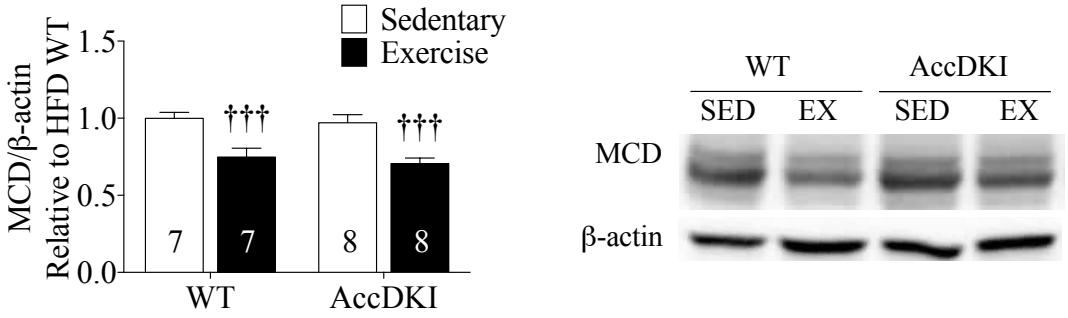

Figure 5.1. Hepatic MCD (MLYCD, Abcam) protein expression (post-hyperinsulinemic euglycemic clamp) in WT and AccDKI mice that were either exercise trained or remained sedentary.

Patients with type 2 diabetes and NAFLD present with impaired mitochondrial respiration [493], while active lifestyles have been associated with improved liver mitochondrial function (as reviewed in Goncalves et al. 2013 [494]). A few studies have shown that exercise improves liver mitochondrial function [495] and/or structure [407] in hepatic steatosis. However, obese individuals have been shown to have higher mitochondrial mass despite lower maximal respiration and hepatic insulin resistance [496]. Therefore, the relationship between hepatic insulin resistance and mitochondrial content/function remains unclear. In Chapter 3, we showed no effect of exercise on the gene expression of markers of mitochondrial function. In addition, protein expression of the electron transport chain (OXPHOS) in the liver showed no differences with exercise training (Figure 5.2). However, OXPHOS is only a measure electron transport chain content and cannot speak to the functionality of the mitochondria. Future studies should measure markers of mitochondrial function to better assess the impact of exercise training on mitochondrial function. For example, a P/O ratio [497] will assess the amount of ATP produced per amount of oxygen consumed in isolated mitochondria. It is therefore a good way to measure mitochondrial efficiency to produce ATP.

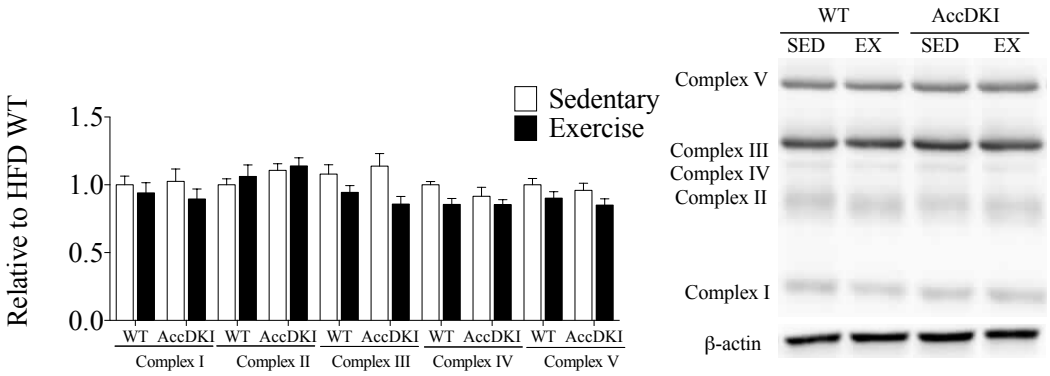

Figure 5.2. Hepatic OXPHOS (Mitosciences) protein expression (post-hyperinsulinemic euglycemic clamp) in WT and AccDKI mice that were exercise trained or remained sedentary.

Obesity has also been associated with elevated levels of mTORC1 and S6K activity in liver, skeletal muscle, and adipose tissue [498–500]. Inhibition of mTORC1 by rapamycin or deletion of S6K removes this inhibitory effect on insulin signaling, in which S6K1 phosphorylates and leads to the degradation of IRS-1 by phosphorylation [501,502]. Whole-body knockout of S6K1 protects mice from developing age- and diet-induced obesity and insulin resistance [500]. In addition, liver-specific deletion of S6K using an adeno-associated virus carrying small hairpin RNA (shRNA) prevents high-fat diet-induced insulin resistance and glucose intolerance independent of body weight [503]. AICAR and exercise have been shown to inhibit the mTORC1 pathway and reduce S6K1 and S6 phosphorylation in skeletal muscle [504–508]. In addition, AMPK activation has also been shown to inhibit the mTORC1 pathway in hepatocytes [509] and

liver [510]. Therefore, this is a potential mechanism by which exercise training may improve hepatic insulin sensitivity independently of ACC phosphorylation or liver lipid content. Future studies examining the importance of liver AMPK and S6K with exercise training and the development of diet-induced insulin resistance are warranted.

In summary, Chapter 3 of this thesis identified that the benefits of exercise training on hepatic insulin sensitivity occur independent of AMPK phosphorylation of ACC1 Ser79 and ACC2 Ser212 and independent of liver lipid levels. Future directions should examine the role of liver mitochondrial function and S6K with exercise training and how this impacts the development of liver insulin sensitivity.

5.4 AMPK, R419, and skeletal muscle insulin sensitivity

R419 is a novel AMPK activator that was shown to increase glucose uptake and fatty acid oxidation in cultured myotubes, effects that were found to be dependent on the expression of AMPK [475]. In addition, when acutely delivered to *db/db* mice, R419 led to an increased flux through both glycolytic and fatty acid oxidative pathways [475]. However, the effects of long-term treatment with R419 on exercise capacity and insulin sensitivity and the importance of AMPK in mediating these effects were not known until our study. Unlike metformin and exercise, in which improvements in hepatic insulin sensitivity were emphasized, R419 was shown to improve peripheral insulin sensitivity by increasing glucose

uptake levels into skeletal muscle, albeit in an AMPK-independent fashion. We found that insulin sensitivity was improved concomitantly with increased protein expression of skeletal muscle GLUT4 in R419-treated mice. In addition, R419 was associated with increased GLUT4 promoter activity [475] suggesting that the effects of R419 on GLUT4 may be the mechanism by which R419 improves skeletal muscle insulin sensitivity. Therefore, a future direction would be to treat GLUT4-MKO mice made obese by high-fat diet with R419 to determine whether increased GLUT4 expression is the means by which R419 improves peripheral glucose disposal [273].

The identification of agents that can mimic exercise for the treatment of metabolic diseases has been an area of interest for some researchers. Additionally, since individuals with type 2 diabetes and obesity present with compromised exercise performance [153], identification of agents that will help improve their ability to exercise would be valuable to improving their daily life. Interestingly, direct AMPK activation with AICAR has been shown to increase activity of skeletal muscle oxidative enzymes [511–513] and to improve exercise performance by ~45% in untrained mice [513]. Because AICAR is not well tolerated in humans, the finding that R419 improved exercise capacity was important. We further showed that R419 treatment increases exercise capacity in skeletal muscle via a skeletal muscle AMPK-dependent mechanism. Since high-fat diet leads to reduced exercise capacity in mice [514], one future direction would be to compare exercise capacity levels pre-treatment with R419 in order to

determine whether R419 is actually improving exercise capacity or whether it is blunting the gradual impairments in exercise capacity that occur with high-fat diet. Therefore, in addition to the insulin sensitizing effects of AMPK activation, future studies examining the potential for AMPK activation in skeletal muscle to improve exercise performance in obese patients is warranted.

5.5 Summary

The main goal of this thesis was to investigate the role of AMPK activation in diet-induced obesity and insulin resistance, the precursor to type 2 diabetes. Three main findings were examined and presented. We show that metformin, the most commonly prescribed anti-diabetic drug improves hepatic insulin resistance by reducing liver lipid levels via inhibition of AMPK phosphorylation of ACC1 Ser79 and ACC2 Ser212 *in vivo*. Future studies should focus on studying the effects of chronic metformin treatment at clinical doses and using diets that represent the typical diet of the obese human populations. Next, exercise-induced improvements in hepatic insulin sensitivity were shown to occur independent of AMPK regulation of ACC1 Ser79 and ACC2 Ser 212, and independent of liver lipid content. Examination of the role of mitochondrial dysfunction and S6K signaling may help elucidate the mechanisms by which exercise training improves hepatic insulin sensitivity. Finally, we found that the novel AMPK activator R419 requires skeletal muscle AMPK to induce improvements in exercise capacity but does not need AMPK to improve

peripheral glucose disposal into skeletal muscle in diet-induced insulin resistance. A future direction is to assess whether R419 improves insulin sensitivity by increasing GLUT4 content in skeletal muscle.

In conclusion, this thesis demonstrates that strategies to activate AMPK in liver and skeletal muscle are associated with improvements in insulin sensitivity. Mechanistically, while metformin treatment leads to reductions in lipid content and improvements in insulin sensitivity, improvements in insulin sensitivity via exercise training and R419 do not depend on lipid content. These findings suggest that future studies examining the effects of AMPK activation in obesity will provide insights into the mechanisms of insulin resistance and will help in the prevention and treatment of obesity and type 2 diabetes.

REFERENCES

1 Ng, M., Fleming, T., Robinson, M., Thomson, B., Graetz, N., Margono, C., Mullany, E. C., Biryukov, S., Abbafati, C., Abera, S. F., et al. (2014) Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet **6736**, 1–16.

2 Ritchie, S. A. and Connell, J. M. C. (2007) The link between abdominal obesity, metabolic syndrome and cardiovascular disease. Nutr. Metab. Cardiovasc. Dis. **17**, 319–326.

3 Alberti, K., George, M., Zimmet, P. and Shaw, J. (2005) The metabolic syndrome - A new worldwide definition. Lancet **366**, 1059–1062.

4 American Diabetes Association. (2014) Standards of medical care in diabetes--2014. Diabetes Care **37**, S14–80.

5 Chan, J. M., Rimm, E. B., Colditz, G. A., Stampfer, M. J. and Willett, W. C. (1994) Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. Diabetes Care **17**, 961–969.

6 Colditz, G. A., Willett, W. C., Rotnitzky, A. and Manson, J. E. (1995) Weight gain as a risk factor for clinical diabetes mellitus in women. Ann. Intern. Med. **122**, 481–486.

7 Shaw, J. E., Sicree, R. A. and Zimmet, P. Z. (2010) Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res. Clin. Pract. **87**, 4–14.

8 WHO. (2014) Diabetes.

9 Seppälä-Lindroos, A., Vehkavaara, S., Häkkinen, A. M., Goto, T., Westerbacka, J., Sovijärvi, A., Halavaara, J. and Yki-Järvinen, H. (2002) Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. J. Clin. Endocrinol. Metab. **87**, 3023–3028.

10 Kim, H. J., Kim, H. J., Lee, K. E., Kim, D. J., Kim, S. K., Ahn, C. W., Lim, S.-K., Kim, K. R., Lee, H. C., Huh, K. B., et al. (2004) Metabolic significance of nonalcoholic fatty liver disease in nonobese, nondiabetic adults. Arch. Intern. Med. **164**, 2169–2175.

11 Clark, J. M. (2006) The epidemiology of nonalcoholic fatty liver disease in adults. J. Clin. Gastroenterol. **40**, S5–S10.

12 Ferreira, V. S. G., Pernambuco, R. B., Lopes, E. P., Morais, C. N., Rodrigues, M. C., Arruda, M. J., Silva, L. M. E. and Vilar, L. (2010) Frequency and risk factors associated with non-alcoholic fatty liver disease in patients with type 2 diabetes mellitus. Arq. Bras. Endocrinol. Metabol. **54**, 362–368.

13 Petersen, K. F., Dufour, S., Feng, J., Befroy, D., Dziura, J., Dalla Man, C., Cobelli, C. and Shulman, G. I. (2006) Increased prevalence of insulin resistance and nonalcoholic fatty liver disease in Asian-Indian men. Proc. Natl. Acad. Sci. U. S. A. **103**, 18273–18277.

14 Wanless, I. R. and Lentz, J. S. (1990) Fatty liver hepatitis (steatohepatitis) and obesity: An autopsy study with analysis of risk factors. Hepatology **12**, 1106–1110.

15 Silverman, J. F., O'Brien, K. F., Long, S., Leggett, N., Khazanie, P. G., Pories, W. J., Norris, H. T. and Caro, J. F. (1990) Liver pathology in morbidly obese patients with and without diabetes. Am. J. Gastroenterol. **85**, 1349–1355.

16 Fabbrini, E., Sullivan, S. and Klein, S. (2010) Obesity and nonalcoholic fatty liver disease: Biochemical, metabolic, and clinical implications. Hepatology **51**, 679–689.

17 Shulman, G. I. (1999) Cellular mechanisms of insulin resistance in humans. Am. J. Cardiol. **84**, 3J–10J.

18 Jornayvaz, F. R. and Shulman, G. I. (2012) Diacylglycerol activation of protein kinase Cε and hepatic insulin resistance. Cell Metab. **15**, 574–584.

19 Sanchez, P. P., Bril, F., Maximos, M., Lomonaco, R., Biernacki, D., Orsak, B., Subbarayan, S., Webb, A., Hecht, J. and K., C. (2015) High Prevalence of Nonalcoholic Fatty Liver Disease in Patients with Type 2 Diabetes Mellitus and Normal Plasma Aminotransferase Levels. J Clin Endocrinol Metab **100**, 2231–2238.

20 Angulo, P. (2002) Nonalcoholic Fatty Liver Disease. N. Engl. J. Med. **346**, 1221–1231.

21 Szczepaniak, L. S., Nurenberg, P., Leonard, D., Browning, J. D., Reingold, J. S., Grundy, S., Hobbs, H. H. and Dobbins, R. L. (2005) Magnetic

resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. Am. J. Physiol. Endocrinol. Metab. **288**, E462–E468.

22 Farrell, G. C. and Larter, C. Z. (2006) Nonalcoholic fatty liver disease: from steatosis to cirrhosis. Hepatology **43**, S99–S112.

23 Brunt, E. M., Janney, C. G., Di Bisceglie, A. M., Neuschwander-Tetri, B. A. and Bacon, B. R. (1999) Nonalcoholic steatohepatitis: A proposal for grading and staging the histological lesions. Am. J. Gastroenterol. **94**, 2467–2474.

24 Saltiel, A. R. and Kahn, C. R. (2001) Insulin signalling and the regulation of glucose and lipid metabolism. Nature **414**, 799–806.

25 Downes, C. P., Gray, A. and Lucocq, J. M. (2005) Probing phosphoinositide functions in signaling and membrane trafficking. Trends Cell Biol. **15**, 259–268.

26 Myers, M. G., Backer, J. M., Sun, X. J., Shoelson, S., Hu, P., Schlessinger, J., Yoakim, M., Schaffhausen, B. and White, M. F. (1992) IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85. Proc. Natl. Acad. Sci. U. S. A. **89**, 10350–10354.

27 Taniguchi, C. M., Emanuelli, B. and Kahn, C. R. (2006) Critical nodes in signalling pathways: insights into insulin action. Nat. Rev. Mol. Cell Biol. **7**, 85–96.

28 Whiteman, E. L., Cho, H. and Birnbaum, M. J. (2002) Role of Akt/protein kinase B in metabolism. Trends Endocrinol. Metab. **13**, 444–451.

29 Cartee, G. D. and Funai, K. (2009) Exercise and insulin: Convergence or divergence at AS160 and TBC1D1? Exerc. Sport Sci. Rev. **37**, 188–195.

30 Ishikura, S. and Klip, A. (2008) Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation. Am. J. Physiol. Cell Physiol. **295**, C1016–C1025.

31 Peck, G. R., Chavez, J. A., Roach, W. G., Budnik, B. A., Lane, W. S., Karlsson, H. K. R., Zierath, J. R. and Lienhard, G. E. (2009) Insulin-stimulated phosphorylation of the rab GTPase-activating protein TBC1D1 regulates GLUT4 translocation. J. Biol. Chem. **284**, 30016–30023.

32 Chen, S., Wasserman, D. H., MacKintosh, C. and Sakamoto, K. (2011) Mice with AS160/TBC1D4-Thr649Ala knockin mutation are glucose intolerant with reduced insulin sensitivity and altered GLUT4 trafficking. Cell Metab. **13**, 68–79.

33 Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature **378**, 785–9.

34 McManus, E. J., Sakamoto, K., Armit, L. J., Ronaldson, L., Shpiro, N., Marquez, R. and Alessi, D. R. (2005) Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. EMBO J. **24**, 1571–1583.

35 Klover, P. J. and Mooney, R. A. (2004) Hepatocytes: Critical for glucose homeostasis. Int. J. Biochem. Cell Biol. **36**, 753–758.

36 Pilkis, S. J. and Granner, D. K. (1992) Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. Annu. Rev. Physiol. **54**, 885–909.

37 Herzig, S., Hedrick, S., Morantte, I., Koo, S.-H., Galimi, F. and Montminy, M. (2003) CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-gamma. Nature **426**, 190–193.

38 Koonen, D. P. Y., Jacobs, R. L., Febbraio, M., Young, M. E., Soltys, C. L. M., Ong, H., Vance, D. E. and Dyck, J. R. B. (2007) Increased hepatic CD36 expression contributes to dyslipidemia associated with diet-induced obesity. Diabetes **56**, 2863–2871.

39 Liu, Y., Dentin, R., Chen, D., Hedrick, S., Ravnskjaer, K., Schenk, S., Milne, J., Meyers, D. J., Cole, P., Yates, J., et al. (2008) A fasting inducible switch modulates gluconeogenesis via activator/coactivator exchange. Nature **456**, 269–273.

40 Puigserver, P., Rhee, J., Donovan, J., Walkey, C. J., Yoon, J. C., Oriente, F., Kitamura, Y., Altomonte, J., Dong, H., Accili, D., et al. (2003) Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. Nature **423**, 550–555.

41 Michael, M. D., Kulkarni, R. N., Postic, C., Previs, S. F., Shulman, G. I., Magnuson, M. A. and Kahn, C. R. (2000) Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. Mol. Cell **6**, 87–97.

42 Yang, L., An, H.-X., Deng, X.-L., Chen, L.-L. and Li, Z.-Y. (2003) Rosiglitazone reverses insulin secretion altered by chronic exposure to free fatty acid via IRS-2-associated phosphatidylinositol 3-kinase pathway. Acta Pharmacol. Sin. **24**, 429–434.

43 Cohen, P., Nimmo, H. G. and Proud, C. G. (1978) How does insulin stimulate glycogen synthesis? Biochem. Soc. Symp. 69–95.

44 Nakae, J., Park, B. C. and Accili, D. (1999) Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a wortmannin-sensitive pathway. J. Biol. Chem. **274**, 15982–15985.

45 Reaven, G. M. (2005) Why Syndrome X? From Harold Himsworth to the Insulin Resistance Syndrome. Cell Metab. **1**, 9–14.

46 Taylor, S. I., Arioglu, E. (1998) Syndromes associated with insulin resistance and acanthosis nigricans. J Basic Clin Physiol Pharmacol **9**, 419–39.

47 Krook, A. and O'Rahilly, S. (1996) Mutant insulin receptors in syndromes of insulin resistance. Baillieres. Clin. Endocrinol. Metab. **10**, 97–122.

48 Millar, C. A., Shewan, A., Hickson, G. R., James, D. E. and Gould, G. W. (1999) Differential regulation of secretory compartments containing the insulin-responsive glucose transporter 4 in 3T3-L1 adipocytes. Mol. Biol. Cell **10**, 3675–3688.

49 Pessin, J. E., Thurmond, D. C., Elmendorf, J. S., Coker, K. J. and Okada, S. (1999) Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location! Location! J. Biol. Chem. **274**, 2593–2596.

50 Rea, S. and James, D. E. (1997) Moving GLUT4: The biogenesis and trafficking of GLUT4 storage vesicles. Diabetes **46**, 1667–1677.

51 Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y. and Satoh, S. (1994) Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature **372**, 182–186.

52 Araki, E., Lipes, M. A., Patti, M.-E., Bruning, J. C., Haag III, B., Johnson, R. S. and Kahn, C. R. (1994) Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature **372**, 186–190.

53 Brüning, J. C., Winnay, J., Bonner-Weir, S., Taylor, S. I., Accili, D. and Kahn, C. R. (1997) Development of a novel polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles. Cell **88**, 561–572.

54 Higaki, Y., Wojtaszewski, J. F. P., Hirshman, M. F., Withers, D. J., Towery, H., White, M. F. and Goodyear, L. J. (1999) Insulin receptor substrate-2 is not necessary for insulin- and exercise- stimulated glucose transport in skeletal muscle. J. Biol. Chem. **274**, 20791–20795.

55 Withers, D. J., Gutierrez, J. S., Towery, H., Burks, D. J., Ren, J. M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G. I., et al. (1998) Disruption of IRS-2 causes type 2 diabetes in mice. Nature **391**, 900–904.

56 Czech, M. P. and Corvera, S. (1999) Signaling mechanisms that regulate glucose transport. J Biol Chem **274**, 1865–1868.

57 Cong, L. N., Chen, H., Li, Y., Zhou, L., McGibbon, M. A., Taylor, S. I. and Quon, M. J. (1997) Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. Mol. Endocrinol. **11**, 1881–1890.

58 Kohn, A. D., Barthel, A., Kovacina, K. S., Boge, A., Wallach, B., Summers, S. A., Birnbaum, M. J., Scot, t P. H., Lawrence, J.C. Jr., Roth, R. A. (1998) Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. J Biol Chem **273**, 11937–11943.

59 Kohn, A. D., Summers, S. A., Birnbaum, M. J. and Roth, R. A. (1996) Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J. Biol. Chem. **271**, 31372–31378.

60 Wang, Q., Somwar, R., Bilan, P. J., Liu, Z., Jin, J., Woodgett, J. R. and Klip, A. (1999) Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. Mol. Cell. Biol. **19**, 4008–4018.

61 DeFronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J. and Felber, J. P. (1981) The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. Diabetes **30**, 1000–1007.

62 DeFronzo, R. A. and Tripathy, D. (2009) Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care **32**, S157–63.

63 Randle, P., Garland, P., Hales, C. and Newsholme, E. (1963) The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet **1**, 785–9.

64 Bowker-Kinley, M. M., Davis, W. I., Wu, P., Harris, R. A. and Popov, K. M. (1998) Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. Biochem. J. **329**, 191–196.

65 Hue, L. and Taegtmeyer, H. (2009) The Randle cycle revisited: a new head for an old hat. Am. J. Physiol. Endocrinol. Metab. **297**, E578–E591.

66 Boden, G., Chen, X., Ruiz, J., White, J. V. and Rossetti, L. (1994) Mechanisms of fatty acid-induced inhibition of glucose uptake. J. Clin. Invest. **93**, 2438–2446.

67 Cline, G. W., Petersen, K. F., Krssak, M., Shen, J., Hundal, R. S., Trajanoski, Z., Inzucchi, S., Dresner, A., Rothman, D. L. and Shulman, G. I. (1999) Impaired glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes. N. Engl. J. Med. **341**, 240–246.

68 Dresner, A., Laurent, D., Marcucci, M., Griffin, M. E., Dufour, S., Cline, G. W., Slezak, L. A., Andersen, D. K., Hundal, R. S., Rothman, D. L., et al. (1999) Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. J. Clin. Invest. **103**, 253–259.

69 Ciaraldi, T. P., Abrams, L., Nikoulina, S., Mudaliar, S. and Henry, R. R. (1995) Glucose transport in cultured human skeletal muscle cells. Regulation by insulin and glucose in nondiabetic and non-insulin-dependent diabetes mellitus subjects. J. Clin. Invest. **96**, 2820–2827.

70 Garvey, W. T., Maianu, L., Zhu, J. H., Brechtel-Hook, G., Wallace, P. and Baron, A. D. (1998) Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. J. Clin. Invest. **101**, 2377–2386.

71 Folli, F., Saad, M. J., Backer, J. M. and Kahn, C. R. (1993) Regulation of phosphatidylinositol 3-kinase activity in liver and muscle of animal models of insulin-resistant and insulin-deficient diabetes mellitus. J. Clin. Invest. **92**, 1787–1794.

72 Saad, M. J. A., Folli, F., Kahn, J. A. and Kahn, C. R. (1993) Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-

kinase in liver and muscle of dexamethasone-treated rats. J. Clin. Invest. **92**, 2065–2072.

73 Yu, C., Chen, Y., Cline, G. W., Zhang, D., Zong, H., Wang, Y., Bergeron, R., Kim, J. K., Cushman, S. W., Cooney, G. J., et al. (2002) Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. J. Biol. Chem. **277**, 50230–50236.

74 Chen, M. T., Kaufman, L. N., Spennetta, T. and Shrago, E. (1992) Effects of high fat-feeding to rats on the interrelationship of body weight, plasma insulin, and fatty acyl-coenzyme A esters in liver and skeletal muscle. Metabolism. **41**, 564–569.

75 Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. **9**, 484–496.

76 Takayama, S., White, M. F. and Kahn, C. R. (1988) Phorbol ester-induced serine phosphorylation of the insulin receptor decreases its tyrosine kinase activity. J Biol Chem **263**, 3440–7.

77 Pillay, T., Whittaker, J. and Siddle, K. (1990) Phorbol ester-induced downregulation of protein kinase C potentiates insulin receptor tyrosine autophosphorylation: evidence for a major constitutive role in insulin receptor regulation. Biochem. Soc. Trans. **18**, 494–5.

78 Lewis, R. E., Cao, L., Perregaux, D. and Czech, M. P. (1990) Threonine 1336 of the human insulin receptor is a major target for phosphorylation by protein kinase C. Biochemistry **29**, 1807–1813.

79 Anderson, C. M. and Olefsky, J. M. (1991) Phorbol ester-mediated protein kinase C interaction with wild-type and COOH-terminal truncated insulin receptors. J. Biol. Chem. **266**, 21760–21764.

80 Gao, Z., Hwang, D., Bataille, F., Lefevre, M., York, D., Quon, M. J. and Ye, J. (2002) Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. J. Biol. Chem. **277**, 48115–48121.

81 Aguirre, V., Werner, E. D., Giraud, J., Lee, Y. H., Shoelson, S. E. and White, M. F. (2002) Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J. Biol. Chem. **277**, 1531–1537.

82 Steinberg, G. R., Smith, A. C., Wormald, S., Malenfant, P., Collier, C. and Dyck, D. J. (2004) Endurance training partially reverses dietary-induced leptin resistance in rodent skeletal muscle. Am. J. Physiol. Endocrinol. Metab. **286**, E57–E63.

83 Bell, K. S., Schmitz-Peiffer C, Lim-Fraser M, Biden TJ, Cooney GJ, K. E. (2000) Acute reversal of lipid-induced muscle insulin resistance is associated with rapid alteration in PKC-theta localization. Am J Physiol Endocrinol Metab **279**, E1196–201.

84 Itani, S. I., Zhou, Q., Pories, W. J., MacDonald, K. G. and Dohm, G. L. (2000) Involvement of protein kinase C in human skeletal muscle insulin resistance and obesity. Diabetes **49**, 1353–1358.

85 Kim, J. K., Fillmore, J. J., Sunshine, M. J., Albrecht, B., Higashimori, T., Kim, D. W., Liu, Z. X., Soos, T. J., Cline, G. W., O'Brien, W. R., et al. (2004) PKC-theta knockout mice are protected from fat-induced insulin resistance. J. Clin. Invest. **114**, 823–827.

86 Arkan, M. C., Hevener, A. L., Greten, F. R., Maeda, S., Li, Z.-W., Long, J. M., Wynshaw-Boris, A., Poli, G., Olefsky, J. and Karin, M. (2005) IKK-beta links inflammation to obesity-induced insulin resistance. Nat. Med. **11**, 191–198.

87 Yuan, M., Konstantopoulos, N., Lee, J., Hansen, L., Li, Z. W., Karin, M. and Shoelson, S. E. (2001) Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science **293**, 1673–1677.

88 Hirosumi, J., Tuncman, G., Chang, L., Görgün, C. Z., Uysal, K. T., Maeda, K., Karin, M. and Hotamisligil, G. S. (2002) A central role for JNK in obesity and insulin resistance. Nature **420**, 333–336.

89 Steinberg, G. R., McAinch, A. J., Chen, M. B., O'Brien, P. E., Dixon, J. B., Cameron-Smith, D. and Kemp, B. E. (2006) The suppressor of cytokine signaling 3 inhibits leptin activation of AMP-kinase in cultured skeletal muscle of obese humans. J. Clin. Endocrinol. Metab. **91**, 3592–3597.

90 Watt, M. J., Dzamko, N., Thomas, W. G., Rose-John, S., Ernst, M., Carling, D., Kemp, B. E., Febbraio, M. A. and Steinberg, G. R. (2006) CNTF reverses obesity-induced insulin resistance by activating skeletal muscle AMPK. Nat. Med. **12**, 541–548.

91 Sabio, G., Kennedy, N. J., Cavanagh-Kyros, J., Jung, D. Y., Ko, H. J., Ong, H., Barrett, T., Kim, J. K. and Davis, R. J. (2010) Role of muscle c-Jun NH2-terminal kinase 1 in obesity-induced insulin resistance. Mol. Cell. Biol. **30**, 106–115.

92 Jorgensen, S. B., O'Neill, H. M., Sylow, L., Honeyman, J., Hewitt, K. A., Palanivel, R., Fullerton, M. D., Öberg, L., Balendran, A., Galic, S., et al. (2013) Deletion of skeletal muscle SOCS3 prevents insulin resistance in obesity. Diabetes **62**, 56–64.

93 Schmitz-Peiffer, C. (2000) Signalling aspects of insulin resistance in skeletal muscle: Mechanisms induced by lipid oversupply. Cell. Signal. **12**, 583–594.

94 Schmitz-Peiffer, C., Browne, C. L., Oakes, N. D., Watkinson, A., Chisholm, D. J., Kraegen, E. W. and Biden, T. J. (1997) Alterations in the expression and cellular localization of protein kinase C isozymes epsilon and theta are associated with insulin resistance in skeletal muscle of the high-fat-fed rat. Diabetes **46**, 169–178.

95 Griffin, M. E., Marcucci, M. J., Cline, G. W., Bell, K., Barucci, N., Lee, D., Goodyear, L. J., Kraegen, E. W., White, M. F. and Shulman, G. I. (1999) Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. Diabetes **48**, 1270–1274.

96 Blouin, C. M., Prado, C., Takane, K. K., Lasnier, F., Garcia-Ocana, A., Ferré, P., Dugail, I. and Hajduch, E. (2010) Plasma membrane subdomain compartmentalization contributes to distinct mechanisms of ceramide action on insulin signaling. Diabetes **59**, 600–610.

97 Fox, T. E., Houck, K. L., O'Neill, S. M., Nagarajan, M., Stover, T. C., Pomianowski, P. T., Unal, O., Yun, J. K., Naides, S. J. and Kester, M. (2007) Ceramide recruits and activates protein kinase C zeta (PKC zeta) within structured membrane microdomains. J. Biol. Chem. **282**, 12450–12457.

98 Galic, S., Oakhill, J. S., Steinberg, G. R. (2010) Adipose tissue as an endocrine organ. Mol Coll Endocrinol **316**, 129–39.

99 Hotamisligil, G. S. (2006) Inflammation and metabolic disorders 1. Nature **444**, 860–867.

100 Olefsky, J. M. and Glass, C. K. (2010) Macrophages, inflammation, and insulin resistance. Annu. Rev. Physiol. **72**, 219–246.

101 Steinberg, G. R. (2007) Inflammation in obesity is the common link between defects in fatty acid metabolism and insulin resistance. Cell Cycle **6**, 888–894.

102 Ueki, K., Kondo, T., Kahn, C. R. (2004) Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Mol. Cell. Biol. **24**, 5434–46.

103 Uysal, K. T., Wiesbrock, S. M., Marino, M. W. and Hotamisligil, G. S. (1997) Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature **389**, 610–614.

104 Wang, Q., Miyakawa, Y., Fox, N. and Kaushansky, K. (2000) Interferon-alpha directly represses megakaryopoiesis by inhibiting thrombopoietin-induced signaling through induction of SOCS-1. Blood **96**, 2093–2099.

105 Shi, H., Tzameli, I., Bjørbæk, C. and Flier, J. S. (2004) Suppressor of cytokine signaling 3 is a physiological regulator of adipocyte insulin signaling. J. Biol. Chem. **279**, 34733–34740.

106 Emanuelli, B., Peraldi, P., Filloux, C., Chavey, C., Freidinger, K., Hilton, D. J., Hotamisligil, G. S. and Van Obberghen, E. (2001) SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. J. Biol. Chem. **276**, 47944–47949.

107 Rieusset, J., Bouzakri, K., Chevillotte, E., Ricard, N., Jacquet, D., Bastard, J.-P., Laville, M. and Vidal, H. (2004) Suppressor of Cytokine Signaling 3 Expression and Insulin Resistance in Skeletal Muscle of Obese and Type 2 Diabetic Patients. Diabetes **53**, 2232–2241.

108 Senn, J. J., Klover, P. J., Nowak, I. A., Zimmers, T. A., Koniaris, L. G., Furlanetto, R. W. and Mooney, R. A. (2003) Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. J. Biol. Chem. **278**, 13740–13746.

109 Steinberg, G. R., Watt, M. J. and Febbraio, M. A. (2009) Cytokine Regulation of AMPK signalling. Front. Biosci. **14**, 1902–1916.

110 Boden, G. (2006) Fatty acid-induced inflammation and insulin resistance in skeletal muscle and liver. Curr. Diab. Rep. **6**, 177–181.

111 Perry, R. J., Samuel, V. T., Petersen, K. F. and Shulman, G. I. (2014) The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. Nature **510**, 84–91.

112 Browning, J. D. and Horton, J. D. (2004) Molecular mediators of hepatic steatosis and liver injury. J. Clin. Invest. **114**, 147–152.

113 Marchesini, G., Brizi, M., Morselli-Labate, A. M., Bianchi, G., Bugianesi, E., McCullough, A. J., Forlani, G. and Melchionda, N. (1999) Association of nonalcoholic fatty liver disease with insulin resistance. Am. J. Med. **107**, 450–455.

114 Samuel, V. T., Liu, Z.-X., Qu, X., Elder, B. D., Bilz, S., Befroy, D., Romanelli, A. J. and Shulman, G. I. (2004) Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. J. Biol. Chem. **279**, 32345–32353.

115 Buettner, R., Ottinger, I., Schölmerich, J. and Bollheimer, L. C. (2004) Preserved direct hepatic insulin action in rats with diet-induced hepatic steatosis. Am. J. Physiol. Endocrinol. Metab. **286**, E828–E833.

116 Bugianesi, E., Gentilcore, E., Manini, R., Natale, S., Vanni, E., Villanova, N., David, E., Rizzetto, M. and Marchesini, G. (2005) A randomized controlled trial of metformin versus vitamin E or prescriptive diet in nonalcoholic fatty liver disease. Am. J. Gastroenterol. **100**, 1082–1090.

117 Cusi, K. (2009) Role of Insulin Resistance and Lipotoxicity in Non-Alcoholic Steatohepatitis. Clin. Liver Dis. **13**, 545–563.

118 Gastaldelli, A., Harrison, S. A., Belfort-Aguilar, R., Hardies, L. J., Balas, B., Schenker, S. and Cusi, K. (2009) Importance of changes in adipose tissue insulin resistance to histological response during thiazolidinedione treatment of patients with nonalcoholic steatohepatitis. Hepatology **50**, 1087–1093.

119 Lonardo, A., Lombardini, S., Scaglioni, F., Carulli, L., Ricchi, M., Ganazzi, D., Adinolfi, L. E., Ruggiero, G., Carulli, N. and Loria, P. (2006) Hepatic steatosis and insulin resistance: Does etiology make a difference? J. Hepatol. **44**, 190–196.

120 Minehira, K., Young, S. G., Villanueva, C. J., Yetukuri, L., Oresic, M., Hellerstein, M. K., Farese, R. V, Horton, J. D., Preitner, F., Thorens, B., et al. (2008) Blocking VLDL secretion causes hepatic steatosis but does not affect peripheral lipid stores or insulin sensitivity in mice. J. Lipid Res. **49**, 2038–2044.

121 Monetti, M., Levin, M. C., Watt, M. J., Sajan, M. P., Marmor, S., Hubbard, B. K., Stevens, R. D., Bain, J. R., Newgard, C. B., Farese, R. V., et al. (2007) Dissociation of Hepatic Steatosis and Insulin Resistance in Mice Overexpressing DGAT in the Liver. Cell Metab. **6**, 69–78.

122 Sanyal, A. J., Campbell-Sargent, C., Mirshahi, F., Rizzo, W. B., Contos, M. J., Sterling, R. K., Luketic, V. A., Shiffman, M. L. and Clore, J. N. (2001) Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterology **120**, 1183–1192.

123 Szendroedi, J., Chmelik, M., Schmid, A. I., Nowotny, P., Brehm, A., Krssak, M., Moser, E. and Roden, M. (2009) Abnormal hepatic energy homeostasis in type 2 diabetes. Hepatology **50**, 1079–1086.

124 Thamer, C., Machann, J., Stefan, N., Haap, M., Schäfer, S., Brenner, S., Kantartzis, K., Claussen, C., Schick, F., Haring, H., et al. (2007) High visceral fat mass and high liver fat are associated with resistance to lifestyle intervention. Obesity (Silver Spring). **15**, 531–538.

125 Kelley, D. E., Goodpaster, B. H. and Storlien, L. (2002) Muscle triglyceride and insulin resistance. Annu. Rev. Nutr. **22**, 325–346.

126 Taira, S., Shimabukuro, M., Higa, M., Yabiku, K., Kozuka, C., Ueda, R., Sunagawa, S., Ohshiro, Y., Doi, M., Nanba, T., et al. (2013) Lipid Deposition in Various Sites of the Skeletal Muscles and Liver Exhibits a Positive Correlation with Visceral Fat Accumulation in Middle-aged Japanese Men with Metabolic Syndrome. Intern. Med. **52**, 1561–1571.

127 Fishbein, M. H., Mogren, C., Gleason, T. and Stevens, W. R. (2006) Relationship of hepatic steatosis to adipose tissue distribution in pediatric nonalcoholic fatty liver disease. J. Pediatr. Gastroenterol. Nutr. **42**, 83–88.

128 Eguchi, Y., Eguchi, T., Mizuta, T., Ide, Y., Yasutake, T., Iwakiri, R., Hisatomi, A., Ozaki, I., Yamamoto, K., Kitajima, Y., et al. (2006) Visceral fat accumulation and insulin resistance are important factors in nonalcoholic fatty liver disease. J. Gastroenterol. **41**, 462–469.

129 Fenkci, S., Rota, S., Sabir, N. and Akdag, B. (2007) Ultrasonographic and biochemical evaluation of visceral obesity in obese women with non-alcoholic fatty liver disease. Eur J Med Res **12**, 68–73.

130 Bjorntorp, P. (1990) Portal adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. Arteriosclerosis **10**, 493–496.

131 Magnusson, I., Rothman, D. L., Katz, L. D., Shulman, R. G. and Shulman, G. I. (1992) Increased rate of gluconeogenesis in type II diabetes mellitus. A 13C nuclear magnetic resonance study. J. Clin. Invest. **90**, 1323–1327.

132 Krssak, M., Brehm, A., Bernroider, E., Anderwald, C., Nowotny, P., Dalla Man, C., Cobelli, C., Cline, G. W., Shulman, G. I., Waldhäusl, W., et al. (2004) Alterations in postprandial hepatic glycogen metabolism in type 2 diabetes. Diabetes **53**, 3048–3056.

133 Samuel, V. T., Liu, Z. X., Qu, X., Elder, B. D., Bilz, S., Befroy, D., Romanelli, A. J. and Shulman, G. I. (2004) Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. J Biol Chem **279**, 32345–32353.

134 Zhang, D., Liu, Z.-X., Choi, C. S., Tian, L., Kibbey, R., Dong, J., Cline, G. W., Wood, P. A. and Shulman, G. I. (2007) Mitochondrial dysfunction due to long-chain Acyl-CoA dehydrogenase deficiency causes hepatic steatosis and hepatic insulin resistance. Proc. Natl. Acad. Sci. U. S. A. **104**, 17075–17080.

135 Varela, G. M., Antwi, D. A., Dhir, R., Yin, X., Singhal, N. S., Graham, M. J., Crooke, R. M. and Ahima, R. S. (2008) Inhibition of ADRP prevents diet-induced insulin resistance. Am. J. Physiol. Gastrointest. Liver Physiol. **295**, G621–G628.

136 Savage, D. B., Petersen, K. F. and Shulman, G. I. (2007) Disordered lipid metabolism and the pathogenesis of insulin resistance. Physiol. Rev. **87**, 507–520.

137 Matsuzaka, T., Shimano, H., Yahagi, N., Kato, T., Atsumi, A., Yamamoto, T., Inoue, N., Ishikawa, M., Okada, S., Ishigaki, N., et al. (2007) Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance. Nat. Med. **13**, 1193–1202.

138 Choi, C. S., Savage, D. B., Abu-Elheiga, L., Liu, Z.-X., Kim, S., Kulkarni, A., Distefano, A., Hwang, Y.-J., Reznick, R. M., Codella, R., et al. (2007) Continuous fat oxidation in acetyl-CoA carboxylase 2 knockout mice

increases total energy expenditure, reduces fat mass, and improves insulin sensitivity. Proc. Natl. Acad. Sci. U. S. A. **104**, 16480–16485.

139 Crooke, S. T. (2000) Progress in antisense technology: The end of the beginning. Methods Enzymol. **313**, 3–45.

140 Kumashiro, N., Erion, D. M., Zhang, D., Kahn, M., Beddow, S. a, Chu, X., Still, C. D., Gerhard, G. S., Han, X., Dziura, J., et al. (2011) Cellular mechanism of insulin resistance in nonalcoholic fatty liver disease. Proc. Natl. Acad. Sci. U. S. A. **108**, 16381–5.

141 Fu, S., Watkins, S. M. and Hotamisligil, G. S. (2012) The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling. Cell Metab. **15**, 623–634.

142 Clouston, A. D. and Powell, E. E. (2004) Nonalcoholic fatty liver disease: Is all the fat bad? Intern. Med. J. **34**, 187–191.

143 Galic, S., Sachithanandan, N., Kay, T. W. and Steinberg, G. R. (2014) Suppressor of cytokine signalling (SOCS) proteins as guardians of inflammatory responses critical for regulating insulin sensitivity. Biochem. J. **461**, 177–188.

144 Torisu, T., Sato, N., Yoshiga, D., Kobayashi, T., Yoshioka, T., Mori, H., Iida, M. and Yoshimura, A. (2007) The dual function of hepatic SOCS3 in insulin resistance in vivo. Genes to Cells **12**, 143–154.

145 Sachithanandan, N., Fam, B. C., Fynch, S., Dzamko, N., Watt, M. J., Wormald, S., Honeyman, J., Galic, S., Proietto, J., Andrikopoulos, S., et al. (2010) Liver-specific suppressor of cytokine signaling-3 deletion in mice enhances hepatic insulin sensitivity and lipogenesis resulting in fatty liver and obesity. Hepatology **52**, 1632–1642.

146 Petersen, K. F., Dufour, S., Befroy, D., Lehrke, M., Hendler, R. E. and Shulman, G. I. (2005) Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. Diabetes **54**, 603–608.

147 Venables, M. C. and Jeukendrup, A. E. (2009) Physical inactivity and obesity: Links with insulin resistance and type 2 diabetes mellitus. Diabetes. Metab. Res. Rev. **25**, S18–23.

148 Admiraal, W. M., van Valkengoed, I. G. M., L de Munter, J. S., Stronks, K., Hoekstra, J. B. L. and Holleman, F. (2011) The association of physical

inactivity with Type 2 diabetes among different ethnic groups. Diabet. Med. **28**, 668–672.

149 Amati, F., Dubé, J. J., Coen, P. M., Stefanovic-Racic, M., Toledo, F. G. S. and Goodpaster, B. H. (2009) Physical inactivity and obesity underlie the insulin resistance of aging. Diabetes Care **32**, 1547–1549.

150 Hawley, J. A. (2004) Exercise as a therapeutic intervention for the prevention and treatment of insulin resistance. Diabetes. Metab. Res. Rev. **20**, 383–393.

151 Devlin, J. T., Hirshman, M., Horton, E. D. and Horton, E. S. (1987) Enhanced peripheral and splanchnic insulin sensitivity in NIDDM men after single bout of exercise. Diabetes **36**, 434–439.

152 Perseghin, G., Price, T. B., Petersen, K. F., Roden, M., Cline, G. W., Gerow, K., Rothman, D. L. and Shulman, G. I. (1996) Increased glucose transport-phosphorylation and muscle glycogen synthesis after exercise training in insulin-resistant subjects. N. Engl. J. Med. **335**, 1357–1362.

153 Ruderman, N. B. and Dean, D. (1998) Malonyl CoA, long chain fatty acyl CoA and insulin resistance in skeletal muscle. J. Basic Clin. Physiol. Pharmacol. **9**, 295–308.

154 Sriwijitkamol, A., Coletta, D. K., Wajcberg, E., Balbontin, G. B., Reyna, S. M., Barrientes, J., Eagan, P. A., Jenkinson, C. P., Cersosimo, E., DeFronzo, R. A., et al. (2007) Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes: A time-course and dose-response study. Diabetes **56**, 836–848.

155 Bandyopadhyay, G. K., Yu, J. G., Ofrecio, J. and Olefsky, J. M. (2006) Increased malonyl-CoA levels in muscle from obese and type 2 diabetic subjects lead to decreased fatty acid oxidation and increased lipogenesis; thiazolidinedione treatment reverses these defects. Diabetes **55**, 2277–2285.

156 Xu, X. J., Pories, W. J., Dohm, L. G. and Ruderman, N. B. (2013) What distinguishes adipose tissue of severely obese humans who are insulin sensitive and resistant? Curr. Opin. Lipidol. **24**, 49–56.

157 Kola, B., Christ-Crain, M., Lolli, F., Arnaldi, G., Giacchetti, G., Boscaro, M., Grossman, A. B. and Korbonits, M. (2008) Changes in adenosine 5′-monophosphate-activated protein kinase as a mechanism of visceral obesity in Cushing's syndrome. J. Clin. Endocrinol. Metab. **93**, 4969–4973.

158 Gauthier, M.-S., O'Brien, E. L., Bigornia, S., Mott, M., Cacicedo, J. M., Xu, X. J., Gokce, N., Apovian, C. and Ruderman, N. (2011) Decreased AMP-activated protein kinase activity is associated with increased inflammation in visceral adipose tissue and with whole-body insulin resistance in morbidly obese humans. Biochem. Biophys. Res. Commun. **404**, 382–387.

159 Winder, W. W. and Hardie, D. G. (1996) Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. Am. J. Physiol. **270**, E299–E304.

160 Vavvas, D., Apazidis, A., Saha, A. K., Gamble, J., Patel, A., Kemp, B. E., Witters, L. A. and Ruderman, N. B. (1997) Contraction-induced changes in acetyl-CoA carboxylase and 5'-AMP-activated kinase in skeletal muscle. J. Biol. Chem. **272**, 13255–13261.

161 Chen, Z. P., McConell, G. K., Michell, B. J., Snow, R. J., Canny, B. J. and Kemp, B. E. (2000) AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. Am. J. Physiol. Endocrinol. Metab. **279**, E1202–E1206.

162 Gibala, M. J., McGee, S. L., Garnham, A. P., Howlett, K. F., Snow, R. J. and Hargreaves, M. (2009) Brief intense interval exercise activates AMPK and p38 MAPK signaling and increases the expression of PGC-1alpha in human skeletal muscle. J. Appl. Physiol. **106**, 929–934.

163 Stephens, T. J., Chen, Z.-P., Canny, B. J., Michell, B. J., Kemp, B. E. and McConell, G. K. (2002) Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise. Am. J. Physiol. Endocrinol. Metab. **282**, E688–E694.

164 Park, H., Kaushik, V. K., Constant, S., Prentki, M., Przybytkowski, E., Ruderman, N. and Saha, A. K. (2002) Coordinate regulation of malonyl-CoA decarboxylase, sn-glycerol-3-phosphate acyltransferase, and acetyl-CoA carboxylase by AMP-activated protein kinase in rat tissues in response to exercise. J. Biol. Chem. **277**, 32571–32577.

165 Wojtaszewski, J. F., Nielsen, P., Hansen, B. F., Richter, E. A. and Kiens, B. (2000) Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle. J. Physiol. **528**, 221–226.

166 Takekoshi, K., Fukuhara, M., Quin, Z., Nissato, S., Isobe, K., Kawakami, Y. and Ohmori, H. (2006) Long-term exercise stimulates adenosine

monophosphate-activated protein kinase activity and subunit expression in rat visceral adipose tissue and liver. Metabolism. **55**, 1122–1128.

167 O'Neill, H. M., Maarbjerg, S. J., Crane, J. D., Jeppesen, J., Jorgensen, S. B., Schertzer, J. D., Shyroka, O., Kiens, B., van Denderen, B. J., Tarnopolsky, M. A., et al. (2011) AMP-activated protein kinase (AMPK) β1β2 muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. Proc. Natl. Acad. Sci. **108**, 16092–16097.

168 Boutcher, S. H. (2011) High-intensity intermittent exercise and fat loss. J. Obes. **2011**, 1–10.

169 Witters, L. (2001) The blooming of the French lilac. J Clin Invest **108**, 1105–1107.

170 Hundal, R. S., Krssak, M., Dufour, S., Laurent, D., Lebon, V., Chandramouli, V., Inzucchi, S. E., Schumann, W. C., Petersen, K. F., Landau, B. R., et al. (2000) Mechanism by which metformin reduces glucose production in type 2 diabetes. Diabetes **49**, 2063–2069.

171 Owen, M. R., Doran, E. and Halestrap, A. P. (2000) Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem. J. **348**, 607–614.

172 El-Mir, M. Y., Nogueira, V., Fontaine, E., Avéret, N., Rigoulet, M. and Leverve, X. (2000) Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. J. Biol. Chem. **275**, 223–228.

173 Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., et al. (2001) Role of AMP-activated protein kinase in mechanism of metformin action. J. Clin. Invest. **108**, 1167–1174.

174 Oakhill, J. S., Scott, J. W. and Kemp, B. E. (2012) AMPK functions as an adenylate charge-regulated protein kinase. Trends Endocrinol. Metab. **23**, 125–132.

175 Quinn, J. M. W., Tam, S., Sims, N. A., Saleh, H., McGregor, N. E., Poulton, I. J., Scott, J. W., Gillespie, M. T., Kemp, B. E. and van Denderen, B. J. W. (2010) Germline deletion of AMP-activated protein kinase beta subunits reduces bone mass without altering osteoclast differentiation or function. FASEB J. **24**, 275–285.

176 Carlson, C. A. and Kim, K. H. (1973) Regulation of hepatic acetyl coenzyme A carboxylase by phosphorylation and dephosphorylation. J. Biol. Chem. **248**, 378–380.

177 Beg, Z. H., Allmann, D. W. and Gibson, D. M. (1973) Modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity with cAMP and with protein fractions of rat liver cytosol. Biochem. Biophys. Res. Commun. **54**, 1362–1369.

178 Yeh, L. A., Lee, K. H., Kim, K. H. (1980) Regulation of liver acetyl-CoA carboxylase. Regulation of phosphorylation and inactivation of ACC by the adenylate energy charge. J Biol Chem **255**, 2308–14.

179 Munday, M. R., Carling, D. and Hardie, D. G. (1988) Negative interactions between phosphorylation of acetyl-CoA carboxylase by the cyclic AMP-dependent and AMP-activated protein kinases. FEBS Lett. **235**, 144–148.

180 Carling, D., Clarke, P. R., Zammit, V. A. and Hardie, D. G. (1989) Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. Eur.J.Biochem. **186**, 129–136.

181 Witczak, C. A., Sharoff, C. G. and Goodyear, L. J. (2008) AMP-activated protein kinase in skeletal muscle: from structure and localization to its role as a master regulator of cellular metabolism. Cell. Mol. Life Sci. **65**, 3737–3755.

182 Xiao, B., Heath, R., Saiu, P., Leiper, F. C., Leone, P., Jing, C., Walker, P. A., Haire, L., Eccleston, J. F., Davis, C. T., et al. (2007) Structural basis for AMP binding to mammalian AMP-activated protein kinase. Nature **449**, 496–500.

183 Mahlapuu, M., Johansson, C., Lindgren, K., Hjälm, G., Barnes, B., Krook, A., Zierath, J., Andersson, L. and Marklund, S. (2004) Expression profiling of the gamma-subunit isoforms of AMPK suggests a major role for gamma3 in white skeletal muscle. Am J Physiol Endocrinol Metab **286**, E194–200.

184 Stapleton, D., Mitchelhill, K. I., Gao, G., Widmer, J., Michell, B. J., Teh, T., House, C. M., Fernandez, C. S., Cox, T., Witters, L. A., et al. (1996) Mammalian AMP-activated protein kinase subfamily. J. Biol. Chem. **271**, 611–614.

185 Lihn, A. S., Jessen, N., Pedersen, S. B., Lund, S. and Richelsen, B. (2004) AICAR stimulates adiponectin and inhibits cytokines in adipose tissue. Biochem. Biophys. Res. Commun. **316**, 853–858.

186 Cheung, P. C., Salt, I. P., Davies, S. P., Hardie, D. G. and Carling, D. (2000) Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. Biochem. J. **346**, 659–669.

187 Woods, A., Salt, I., Scott, J., Hardie, D. and Carling, D. (1996) The alpha1 and alpha2 isoforms of the AMPK have similar activities in rat liver but exhibit differences in substrate specificity in vitro. FEBS Lett. **397**, 347–51.

188 Dzamko, N., van Denderen, B. J. W., Hevener, A. L., Jørgensen, S. B., Honeyman, J., Galic, S., Chen, Z. P., Watt, M. J., Campbell, D. J., Steinberg, G. R., et al. (2010) AMPK β1 deletion reduces appetite, preventing obesity and hepatic insulin resistance. J. Biol. Chem. **285**, 115–122.

189 Chen Z1, Heierhorst J, Mann RJ, Mitchelhill KI, Michell BJ, Witters LA, Lynch GS, Kemp BE, S. D. (1999) Expression of the AMP-activated protein kinase beta1 and beta2 subunits in skeletal muscle. FEBS Lett. **460**, 343–8.

190 Thornton, C., Snowden, M. A. and Carling, D. (1998) Identification of a novel AMP-activated protein kinase beta subunit isoform that is highly expressed in skeletal muscle. J Biol Chem **273**, 12443–12450.

191 Steinberg, G. R., O'Neill, H. M., Dzamko, N. L., Galic, S., Naim, T., Koopman, R., Jørgensen, S. B., Honeyman, J., Hewitt, K., Chen, Z.-P., et al. (2010) Whole body deletion of AMP-activated protein kinase β2 reduces muscle AMPK activity and exercise capacity. J. Biol. Chem. **285**, 37198–37209.

192 Luptak, I., Shen, M., He, H., Hirshman, M. F., Musi, N., Goodyear, L. J., Yan, J., Wakimoto, H., Morita, H., Arad, M., et al. (2007) Aberrant activation of AMP-activated protein kinase remodels metabolic network in favor of cardiac glycogen storage. J. Clin. Invest. **117**, 1432–1439.

193 Yu, H., Fujii, N., Hirshman, M. F., Pomerleau, J. M. and Goodyear, L. J. (2004) Cloning and characterization of mouse 5'-AMP-activated protein kinase gamma3 subunit. Am. J. Physiol. Cell Physiol. **286**, C283–C292.

194 Barnes, B. R., Marklund, S., Steiler, T. L., Walter, M., Hjälm, G., Amarger, V., Mahlapuu, M., Leng, Y., Johansson, C., Galuska, D., et al. (2004) The 5'-AMP-activated protein kinase gamma3 isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle. J. Biol. Chem. **279**, 38441–38447.

195 Crute, B. E., Seefeld, K., Gamble, J., Kemp, B. E. and Witters, L. A. (1998) Functional domains of the alpha1 catalytic subunit of the AMP-activated protein kinase. J. Biol. Chem. **273**, 35347–35354.

196 Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D. and Hardie, D. G. (1996) Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. J. Biol. Chem. **271**, 27879–27887.

197 Iseli, T. J., Walter, M., van Denderen, B. J. W., Katsis, F., Witters, L. A., Kemp, B. E., Michell, B. J. and Stapleton, D. (2005) AMP-activated protein kinase beta subunit tethers alpha and gamma subunits via its C-terminal sequence (186-270). J. Biol. Chem. **280**, 13395–13400.

198 Mitchelhill, K. I., Michell, B. J., House, C. M., Stapleton, D., Dyck, J., Gamble, J., Ullrich, C., Witters, L. A. and Kemp, B. E. (1997) Posttranslational modifications of the 5'-AMP-activated protein kinase beta1 subunit. J. Biol. Chem. **272**, 24475–24479.

199 Townley, R. and Shapiro, L. (2007) Crystal structures of the adenylate sensor from fission yeast AMP-activated protein kinase. Science **315**, 1726–1729.

200 Warden, S. M., Richardson, C., O'Donnell, J. J., Stapleton, D., Kemp, B. E. and Witters, L. A. (2001) Post-translational modifications of the beta-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization. Biochem. J. **354**, 275–83.

201 Polekhina, G., Gupta, A., Michell, B. J., Van Denderen, B., Murthy, S., Feil, S. C., Jennings, I. G., Campbell, D. J., Witters, L. A., Parker, M. W., et al. (2003) AMPK β subunit targets metabolic stress sensing to glycogen. Curr. Biol. **13**, 867–871.

202 McBride, A., Ghilagaber, S., Nikolaev, A. and Hardie, D. G. (2009) The Glycogen-Binding Domain on the AMPK beta Subunit Allows the Kinase to Act as a Glycogen Sensor. Cell Metab. **9**, 23–34.

203 Sanders, M. J., Grondin, P. O., Hegarty, B. D., Snowden, M. A. and Carling, D. (2007) Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. Biochem. J. **403**, 139–148.

204 Cool, B., Zinker, B., Chiou, W., Kifle, L., Cao, N., Perham, M., Dickinson, R., Adler, A., Gagne, G., Iyengar, R., et al. (2006) Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. Cell Metab. **3**, 403–416.

205 Scott, J. W., van Denderen, B. J. W., Jorgensen, S. B., Honeyman, J. E., Steinberg, G. R., Oakhill, J. S., Iseli, T. J., Koay, A., Gooley, P. R., Stapleton, D., et al. (2008) Thienopyridone Drugs Are Selective Activators of AMP-Activated Protein Kinase β1-Containing Complexes. Chem. Biol. **15**, 1220–1230.

206 Hawley, S. A., Fullerton, M. D., Ross, F. A., Schertzer, J. D., Chevtzoff, C., Walker, K. J., Peggie, M. W., Zibrova, D., Green, K. A., Mustard, K. J., et al. (2012) The Ancient Drug Salicylate Directly Activates AMP-Activated Protein Kinase. Science (80-.). **336**, 918–922.

207 Lai, Y.-C., Kviklyte, S., Vertommen, D., Lantier, L., Foretz, M., Viollet, B., Hallén, S. and Rider, M. H. (2014) A small-molecule benzimidazole derivative that potently activates AMPK to increase glucose transport in skeletal muscle: Comparison with effects of contraction and other AMPK activators. Biochem. J. **460**, 363–375.

208 Zadra, G., Photopoulos, C., Tyekucheva, S., Heidari, P., Weng, Q. P., Fedele, G., Liu, H., Scaglia, N., Priolo, C., Sicinska, E., et al. (2014) A novel direct activator of AMPK inhibits prostate cancer growth by blocking lipogenesis. EMBO Mol. Med. **6**, 519–538.

209 Oakhill, J. S., Steel, R., Chen, Z.-P., Scott, J. W., Ling, N., Tam, S. and Kemp, B. E. (2011) AMPK is a direct adenylate charge-regulated protein kinase. Science **332**, 1433–1435.

210 Xiao, B., Sanders, M. J., Underwood, E., Heath, R., Mayer, F. V, Carmena, D., Jing, C., Walker, P. A., Eccleston, J. F., Haire, L. F., et al. (2011) Structure of mammalian AMPK and its regulation by ADP. Nature **472**, 230–233.

211 Oakhill, J. S., Chen, Z.-P., Scott, J. W., Steel, R., Castelli, L. A., Ling, N., Macaulay, S. L. and Kemp, B. E. (2010) β-Subunit myristoylation is the

gatekeeper for initiating metabolic stress sensing by AMP-activated protein kinase (AMPK). Proc. Natl. Acad. Sci. U. S. A. **107**, 19237–19241.

212 Gowans, G. J., Hawley, S. A., Ross, F. A. and Hardie, D. G. (2013) AMP is a true physiological regulator of amp-activated protein kinase by both allosteric activation and enhancing net phosphorylation. Cell Metab. **18**, 556–566.

213 Hardie, D. G. (2015) AMPK: positive and negative regulation, and its role in whole-body energy homeostasis. Curr. Opin. Cell Biol. **33**, 1–7.

214 Xiao, B., Sanders, M. J., Carmena, D., Bright, N. J., Haire, L. F., Underwood, E., Patel, B. R., Heath, R. B., Walker, P. a, Hallen, S., et al. (2013) Structural basis of AMPK regulation by small molecule activators. Nat. Commun. **4**, 1–17.

215 Calabrese, M. F., Rajamohan, F., Harris, M. S., Caspers, N. L., Magyar, R., Withka, J. M., Wang, H., Borzilleri, K. A., Sahasrabudhe, P. V., Hoth, L. R., et al. (2014) Structural basis for AMPK activation: Natural and synthetic ligands regulate kinase activity from opposite poles by different molecular mechanisms. Structure **22**, 1161–1172.

216 Hardie, D. G. (2014) AMPK—Sensing Energy while Talking to Other Signaling Pathways. Cell Metab. **20**, 939–952.

217 Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Mäkelä, T. P., Alessi, D. R. and Hardie, D. G. (2003) Complexes between the LKB1 tumor suppressor, STRAD α/β and MO25 α/β are upstream kinases in the AMP-activated protein kinase cascade. J. Biol. **2**, 28.

218 Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A. and Cantley, L. C. (2004) The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc. Natl. Acad. Sci. U. S. A. **101**, 3329–3335.

219 Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G. D., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M. and Carling, D. (2003) LKB1 is the Upstream Kinase in the AMP-Activated Protein Kinase Cascade. Curr. Biol. **13**, 2004–2008.

220 Woods, A., Dickerson, K., Heath, R., Hong, S.-P., Momcilovic, M., Johnstone, S. R., Carlson, M. and Carling, D. (2005) Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab. **2**, 21–33.

221 Hurley, R. L., Anderson, K. A., Franzone, J. M., Kemp, B. E., Means, A. R. and Witters, L. A. (2005) The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. J. Biol. Chem. **280**, 29060–29066.

222 Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B. G. and Hardie, D. G. (2005) Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab. **2**, 9–19.

223 Herrero-Martín, G., Høyer-Hansen, M., García-García, C., Fumarola, C., Farkas, T., López-Rivas, A. and Jäättelä, M. (2009) TAK1 activates AMPK-dependent cytoprotective autophagy in TRAIL-treated epithelial cells. EMBO J **28**, 677–85.

224 Sakamoto, K., Göransson, O., Hardie, D. G. and Alessi, D. R. (2004) Activity of LKB1 and AMPK-related kinases in skeletal muscle: effects of contraction, phenformin, and AICAR. Am. J. Physiol. Endocrinol. Metab. **287**, E310–E317.

225 Zhang, Y. L., Guo, H., Zhang, C. S., Lin, S. Y., Yin, Z., Peng, Y., Luo, H., Shi, Y., Lian, G., Zhang, C., et al. (2013) AMP as a low-energy charge signal autonomously initiates assembly of axin-ampk-lkb1 complex for AMPK activation. Cell Metab. **18**, 546–555.

226 Zhang, C. S., Jiang, B., Li, M., Zhu, M., Peng, Y., Zhang, Y.-L., Wu, Y.-Q., Li, T. Y., Liang, Y., Lu, Z., et al. (2014) The Lysosomal v-ATPase-Ragulator Complex Is a Common Activator for AMPK and mTORC1, Acting as a Switch between Catabolism and Anabolism. Cell Metab. **20**, 526–540.

227 Sahlin, K., Tonkonogi, M. and Söderlund, K. (1998) Energy supply and muscle fatigue in humans. Acta Physiol. Scand. **162**, 261–266.

228 Fujii, N., Hayashi, T., Hirshman, M. F., Smith, J. T., Habinowski, S. A., Kaijser, L., Mu, J., Ljungqvist, O., Birnbaum, M. J., Witters, L. A., et al. (2000) Exercise induces isoform-specific increase in 5'AMP-activated protein kinase activity in human skeletal muscle. Biochem. Biophys. Res. Commun. **273**, 1150–1155.

229 Chen, Z. P., Stephens, T. J., Murthy, S., Canny, B. J., Hargreaves, M., Witters, L. A., Kemp, B. E. and McConell, G. K. (2003) Effect of exercise intensity on skeletal muscle AMPK signaling in humans. Diabetes **52**, 2205–2212.

230 Schaffer, J. E. and Lodish, H. F. (1994) Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. Cell **79**, 427–436.

231 Hayashi, T., Hirshman, M. F., Fujii, N., Habinowski, S. A., Witters, L. A. and Goodyear, L. J. (2000) Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. Diabetes **49**, 527–531.

232 Wojtaszewski, J. F. P., Birk, J. B., Frøsig, C., Holten, M., Pilegaard, H. and Dela, F. (2005) 5′ AMP activated protein kinase expression in human skeletal muscle: Effects of strength training and type 2 diabetes. J. Physiol. **564**, 563–573.

233 Treebak, J. T., Birk, J. B., Hansen, B. F., Olsen, G. S. and Wojtaszewski, J. F. P. (2009) A-769662 activates AMPK beta1-containing complexes but induces glucose uptake through a PI3-kinase-dependent pathway in mouse skeletal muscle. Am. J. Physiol. Cell Physiol. **297**, C1041–C1052.

234 Birk, J. B. and Wojtaszewski, J. F. P. (2006) Predominant alpha2/beta2/gamma3 AMPK activation during exercise in human skeletal muscle. J. Physiol. **577**, 1021–1032.

235 Treebak, J. T., Pehmøller, C., Kristensen, J. M., Kjøbsted, R., Birk, J. B., Schjerling, P., Richter, E. a, Goodyear, L. J. and Wojtaszewski, J. F. P. (2014) Acute exercise and physiological insulin induce distinct phosphorylation signatures on TBC1D1 and TBC1D4 proteins in human skeletal muscle. J. Physiol. **592**, 351–75.

236 Treebak, J. T., Birk, J. B., Rose, A. J., Kiens, B., Richter, E. A. and Wojtaszewski, J. F. P. (2007) AS160 phosphorylation is associated with activation of alpha2beta2gamma1- but not alpha2beta2gamma3-AMPK trimeric complex in skeletal muscle during exercise in humans. Am. J. Physiol. Endocrinol. Metab. **292**, E715–E722.

237 Dzamko, N., Schertzer, J. D., Ryall, J. G., Steel, R., Macaulay, S. L., Wee, S., Chen, Z.-P., Michell, B. J., Oakhill, J. S., Watt, M. J., et al. (2008) AMPK-independent pathways regulate skeletal muscle fatty acid oxidation. J. Physiol. **586**, 5819–5831.

238 Mu, J., Brozinick, J. T., Valladares, O., Bucan, M. and Birnbaum, M. J. (2001) A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. Mol. Cell **7**, 1085–1094.

239 Viollet, B., Andreelli, F., Jørgensen, S. B., Perrin, C., Geloen, A., Flamez, D., Mu, J., Lenzner, C., Baud, O., Bennoun, M., et al. (2003) The AMP-activated protein kinase alpha2 catalytic subunit controls whole-body insulin sensitivity. J. Clin. Invest. **111**, 91–98.

240 Mortensen, B., Poulsen, P., Wegner, L., Stender-Petersen, K. L., Ribel-Madsen, R., Friedrichsen, M., Birk, J. B., Vaag, A. and Wojtaszewski, J. F. P. (2009) Genetic and metabolic effects on skeletal muscle AMPK in young and older twins. Am. J. Physiol. Endocrinol. Metab. **297**, E956–E964.

241 Murphy, R. M. (2011) Enhanced technique to measure proteins in single segments of human skeletal muscle fibers: fiber-type dependence of AMPK-alpha1 and -beta1. J. Appl. Physiol. **110**, 820–825.

242 O'Neill, H. M. (2013) AMPK and exercise: Glucose uptake and insulin sensitivity. Diabetes Metab. J. **37**, 1–21.

243 Friedrichsen, M., Mortensen, B., Pehmøller, C., Birk, J. B. and Wojtaszewski, J. F. P. (2013) Exercise-induced AMPK activity in skeletal muscle: Role in glucose uptake and insulin sensitivity. Mol. Cell. Endocrinol. **366**, 204–214.

244 Musi, N. and Goodyear, L. J. (2003) AMP-activated protein kinase and muscle glucose uptake. Acta Physiol. Scand. **178**, 337–345.

245 Richter, E. A. and Hargreaves, M. (2013) Exercise, GLUT4, and skeletal muscle glucose uptake. Physiol. Rev. **93**, 993–1017.

246 Lantier, L., Fentz, J., Mounier, R., Leclerc, J., Treebak, J. T., Pehmøller, C., Sanz, N., Sakakibara, I., Saint-Amand, E., Rimbaud, S., et al. (2014) AMPK controls exercise endurance, mitochondrial oxidative capacity, and skeletal muscle integrity. FASEB J. **28**, 3211–3224.

247 Sakoda, H., Ogihara, T., Anai, M., Fujishiro, M., Ono, H., Onishi, Y., Katagiri, H., Abe, M., Fukushima, Y., Shojima, N., et al. (2002) Activation of AMPK is essential for AICAR-induced glucose uptake by skeletal muscle but not adipocytes. Am. J. Physiol. Endocrinol. Metab. **282**, E1239–E1244.

248 Iglesias, M. A., Ye, J. M., Frangioudakis, G., Saha, A. K., Tomas, E., Ruderman, N. B., Cooney, G. J. and Kraegen, E. W. (2002) AICAR administration causes an apparent enhancement of muscle and liver insulin action in insulin-resistant high-fat-fed rats. Diabetes **51**, 2886–2894.

249 Bergeron, R., Previs, S. F., Cline, G. W., Perret, P., Russell, R. R., Young, L. H. and Shulman, G. I. (2001) Effect of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside infusion on in vivo glucose and lipid metabolism in lean and obese Zucker rats. Diabetes **50**, 1076–1082.

250 Koistinen, H. A., Galuska, D., Chibalin, A. V., Yang, J., Zierath, J. R., Holman, G. D. and Wallberg-Henriksson, H. (2003) 5-Amino-imidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes. Diabetes **52**, 1066–1072.

251 Jensen, T. E., Rose, A. J., Hellsten, Y., Wojtaszewski, J. F. P. and Richter, E. A. (2007) Caffeine-induced Ca(2+) release increases AMPK-dependent glucose uptake in rodent soleus muscle. Am. J. Physiol. Endocrinol. Metab. **293**, E286–E292.

252 Pehmøller, C., Treebak, J. T., Birk, J. B., Chen, S., Mackintosh, C., Hardie, D. G., Richter, E. A. and Wojtaszewski, J. F. P. (2009) Genetic disruption of AMPK signaling abolishes both contraction- and insulin-stimulated TBC1D1 phosphorylation and 14-3-3 binding in mouse skeletal muscle. Am. J. Physiol. Endocrinol. Metab. **297**, E665–E675.

253 Funai, K. and Cartee, G. D. (2009) Inhibition of contraction-stimulated amp-activated protein kinase inhibits contraction-stimulated increases in pas-tbc1d1 and glucose transport without altering pas-as160 in rat skeletal muscle. Diabetes **58**, 1096–1104.

254 Vichaiwong, K., Purohit, S., An, D., Toyoda, T., Jessen, N., Hirshman, M. F. and Goodyear, L. J. (2010) Contraction regulates site-specific phosphorylation of TBC1D1 in skeletal muscle. Biochem. J. **431**, 311–320.

255 Taylor, E. B., An, D., Kramer, H. F., Yu, H., Fujii, N. L., Roeckl, K. S. C., Bowles, N., Hirshman, M. F., Xie, J., Feener, E. P., et al. (2008) Discovery of TBC1D1 as an insulin-, AICAR-, and contraction-stimulated signaling nexus in mouse skeletal muscle. J. Biol. Chem. **283**, 9787–9796.

256 Musi, N., Fujii, N., Hirshman, M. F., Ekberg, I., Fröberg, S., Ljungqvist, O., Thorell, A. and Goodyear, L. J. (2001) AMP-activated protein kinase (AMPK) is activated in muscle of subjects with type 2 diabetes during exercise. Diabetes **50**, 921–927.

257 Hardie, D. G., Carling, D. and Carlson, M. (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu. Rev. Biochem. **67**, 821–855.

258 Kemp, B. E., Mitchelhill, K. I., Stapleton, D., Michell, B. J., Chen, Z. P. and Witters, L. A. (1999) Dealing with energy demand: the AMP-activated protein kinase. Trends Biochem. Sci. **24**, 22–25.

259 Jørgensen, S. B., Viollet, B., Andreelli, F., Frøsig, C., Birk, J. B., Schjerling, P., Vaulont, S., Richter, E. A., Wojtaszewski, J. F. P. (2004) Knockout of the α2 but Not α1 5′-AMP-activated Protein Kinase Isoform Abolishes 5-Aminoimidazole-4-carboxamide-1-β-4-ribofuranosidebut Not Contraction-induced Glucose Uptake in Skeletal Muscle. J Biol Chem **279**, 1070–1079.

260 Szekeres, F., Chadt, A., Tom, R. Z., Deshmukh, A. S., Chibalin, A. V., Bjornholm, M., Al-Hasani, H. and Zierath, J. R. (2012) The Rab-GTPase-activating protein TBC1D1 regulates skeletal muscle glucose metabolism. AJP Endocrinol. Metab. **303**, E524–E533.

261 Stöckli, J., Meoli, C., Hoffman, N., Fazakerley, D., Pant, H., Cleasby, M., Ma, X., Kleinert, M., Brandon, A., Lopez, J., et al. (2015) The RabGAP TBC1D1 plays a central role in exercise-regulated glucose metabolism in skeletal muscle. Diabetes **64**, 1914–1922.

262 Hällsten, K., Virtanen, K. A., Lönnqvist, F., Sipilä, H., Oksanen, A., Viljanen, T., Rönnemaa, T., Viikari, J., Knuuti, J. and Nuutila, P. (2002) Rosiglitazone but not metformin enhances insulin- and exercise-stimulated skeletal muscle glucose uptake in patients with newly diagnosed type 2 diabetes. Diabetes **51**, 3479–3485.

263 Turban, S., Stretton, C., Drouin, O., Green, C. J., Watson, M. L., Gray, A., Ross, F., Lantier, L., Viollet, B., Hardie, D. G., et al. (2012) Defining the contribution of AMP-activated protein kinase (AMPK) and protein kinase C (PKC) in regulation of glucose uptake by metformin in skeletal muscle cells. J Biol Chem **287**, 20088–20099.

264 Hunter, R. W., Foretz, M., Bultot, L., Fullerton, M. D., Deak, M., Ross, F. A., Hawley, S. A., Shpiro, N., Viollet, B., Barron, D., et al. (2014) Mechanism of action of compound-13: An α1-selective small molecule activator of AMPK. Chem. Biol. **21**, 866–879.

265 Kennedy, J. W., Hirshman, M. F., Gervino, E. V, Ocel, J. V, Forse, R. A., Hoenig, S. J., Aronson, D., Goodyear, L. J. and Horton, E. S. (1999) Acute exercise induces GLUT4 translocation in skeletal muscle of normal human subjects and subjects with type 2 diabetes. Diabetes **48**, 1192–1197.

266 Zheng, D., MacLean, P. S., Pohnert, S. C., Knight, J. B., Olson, A. L., Winder, W. W. and Dohm, G. L. (2001) Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. J. Appl. Physiol. **91**, 1073–1083.

267 Merrill, G. F., Kurth, E. J., Hardie, D. G. and Winder, W. W. (1997) AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am. J. Physiol. **273**, E1107–E1112.

268 Kurth-Kraczek, E. J., Hirshman, M. F., Goodyear, L. J. and Winder, W. W. (1999) 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. Diabetes **48**, 1667–1671.

269 Winder, W. W. and Hardie, D. G. (1999) AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. Am. J. Physiol. **277**, E1–E10.

270 Daugaard, J. R. and Richter, E. A. (2004) Muscle- and fibre type-specific expression of glucose transporter 4, glycogen synthase and glycogen phosphorylase proteins in human skeletal muscle. Pflugers Arch Eur J Physiol **447**, 452–456.

271 Tsao, T. S., Burcelin, R., Katz, E. B., Huang, L. and Charron, M. J. (1996) Enhanced insulin action due to targeted GLUT4 overexpression exclusively in muscle. Diabetes **45**, 28–36.

272 Atkinson, B. J., Griesel, B. A., King, C. D., Josey, M. A. and Olson, A. L. (2013) Moderate GLUT4 overexpression improves insulin sensitivity and fasting triglyceridemia in high-fat diet-fed transgenic mice. Diabetes **62**, 2249–2258.

273 Zisman, A., Peroni, O. D., Abel, E. D., Michael, M. D., Mauvais-Jarvis, F., Lowell, B. B., Wojtaszewski, J. F., Hirshman, M. F., Virkamaki, A., Goodyear, L. J., et al. (2000) Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. Nat. Med. **6**, 924–928.

274 Lima, G. A., Anhê, G. F., Giannocco, G., Nunes, M. T., Correa-Giannella, M. L. and Machado, U. F. (2009) Contractile activity per se induces transcriptional activation of SLC2A4 gene in soleus muscle: involvement of MEF2D, HIF-1a, and TRalpha transcriptional factors. Am. J. Physiol. Endocrinol. Metab. **296**, E132–E138.

275 McGee, S. L., Van Denderen, B. J. W., Howlett, K. F., Mollica, J., Schertzer, J. D., Kemp, B. E. and Hargreaves, M. (2008) AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. Diabetes **57**, 860–867.

276 Kawanaka, K., Nolte, L. A., Han, D. H., Hansen, P. A. and Holloszy, J. O. (2000) Mechanisms underlying impaired GLUT-4 translocation in glycogen-supercompensated muscles of exercised rats. Am. J. Physiol. Endocrinol. Metab. **279**, E1311–E1318.

277 Holmes, B. F., Kurth-Kraczek, E. J. and Winder, W. W. (1999) Chronic activation of 5'-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. J. Appl. Physiol. **87**, 1990–1995.

278 Fujii, N., Hirshman, M. F., Kane, E. M., Ho, R. C., Peter, L. E., Seifert, M. M. and Goodyear, L. J. (2005) AMP-activated protein kinase alpha2 activity is not essential for contraction- and hyperosmolarity-induced glucose transport in skeletal muscle. J. Biol. Chem. **280**, 39033–39041.

279 Jørgensen, S. B., Nielsen, J. N., Birk, J. B., Olsen, G. S., Viollet, B., Andreelli, F., Schjerling, P., Vaulont, S., Hardie, D. G., Hansen, B. F., et al. (2004) The α2-5′ AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. Diabetes **53**, 3074–3081.

280 Lee-Young, R. S., Griffee, S. R., Lynes, S. E., Bracy, D. P., Ayala, J. E., McGuinness, O. P. and Wasserman, D. H. (2009) Skeletal muscle AMP-activated protein kinase is essential for the metabolic response to exercise in vivo. J. Biol. Chem. **284**, 23925–23934.

281 Lee-Young, R. S., Ayala, J. E., Fueger, P. T., Mayes, W. H., Kang, L. and Wasserman, D. H. (2011) Obesity impairs skeletal muscle AMPK signaling during exercise: role of AMPKα2 in the regulation of exercise capacity in vivo. Int. J. Obes. (Lond). **35**, 982–989.

282 Jørgensen, S. B., Wojtaszewski, J. F. P., Viollet, B., Andreelli, F., Birk, J. B., Hellsten, Y., Schjerling, P., Vaulont, S., Neufer, P. D., Richter, E. A., et al. (2005) Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle. FASEB J. **19**, 1146–1148.

283 Jørgensen, S. B., Treebak, J. T., Viollet, B., Schjerling, P., Vaulont, S., Wojtaszewski, J. F. P. and Richter, E. A. (2007) Role of AMPKalpha2 in basal, training-, and AICAR-induced GLUT4, hexokinase II, and

mitochondrial protein expression in mouse muscle. Am. J. Physiol. Endocrinol. Metab. **292**, E331–E339.

284 Schertzer, J., Gehrig, S., Ryall, J. and Lynch, G. (2007) Modulation of insulin-like growth factor (IGF)-I and IGF-binding protein interactions enhances skeletal muscle regeneration and ameliorates the dystrophic pathology in mdx mice. Am J Pathol **171**, 1180–8.

285 Russell, R. R., Li, J., Coven, D. L., Pypaert, M., Zechner, C., Palmeri, M., Giordano, F. J., Mu, J., Birnbaum, M. J. and Young, L. H. (2004) AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. J. Clin. Invest. **114**, 495–503.

286 Gianni, P., Jan, K. J., Douglas, M. J., Stuart, P. M. and Tarnopolsky, M. A. (2004) Oxidative stress and the mitochondrial theory of aging in human skeletal muscle. Exp. Gerontol. **39**, 1391–1400.

287 Agbulut, O., Noirez, P., Beaumont, F. and Butler-Browne, G. (2003) Myosin heavy chain isoforms in postnatal muscle development of mice. Biol. Cell **95**, 399–406.

288 Thomas, M. M., Wang, D. C., D'Souza, D. M., Krause, M. P., Layne, A. S., Criswell, D. S., O'Neill, H. M., Connor, M. K., Anderson, J. E., Kemp, B. E., et al. (2014) Muscle-specific AMPK β1β2-null mice display a myopathy due to loss of capillary density in nonpostural muscles. FASEB J. **28**, 2098–2107.

289 Holloszy, J. O. (1967) Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. J. Biol. Chem. **242**, 2278–2282.

290 Hoppeler, H., Luthi, P., Claassen, H., Weibel, E.R., Howald, H. (1973) The ultrastructure of the normal human skeletal muscle. A morphometric analysis on untrained men, women and well-trained orienteers. Pflugers Arch **28**, 217–232.

291 Gollnick, P.D., Armstrong, R.B., Saltin, B., Saubert IV, C.W., Sembrowich, W.L., Shepherd, R. E. (1973) Effect of training on enzyme activity and fiber composition of human skeletal muscle. J. Appl. Physiol. **34**, 107–112.

292 Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R. C., et al. (1999)

Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell **98**, 115–124.

293 Zechner, C., Lai, L., Zechner, J. F., Geng, T., Yan, Z., Rumsey, J. W., Collia, D., Chen, Z., Wozniak, D. F., Leone, T. C., et al. (2010) Total skeletal muscle PGC-1 deficiency uncouples mitochondrial derangements from fiber type determination and insulin sensitivity. Cell Metab. **12**, 633–642.

294 Terada, S., Goto, M., Kato, M., Kawanaka, K., Shimokawa, T. and Tabata, I. (2002) Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. Biochem. Biophys. Res. Commun. **296**, 350–354.

295 Barré, L., Richardson, C., Hirshman, M. F., Brozinick, J., Fiering, S., Kemp, B. E., Goodyear, L. J. and Witters, L. A. (2007) Genetic model for the chronic activation of skeletal muscle AMP-activated protein kinase leads to glycogen accumulation. Am. J. Physiol. Endocrinol. Metab. **292**, E802–E811.

296 Rockl, K. S. C., Hirshman, M. F., Brandauer, J., Fujii, N., Witters, L. A. and Goodyear, L. J. (2007) Skeletal muscle adaptation to exercise training - AMP-activated protein kinase mediates muscle fiber type shift. Diabetes **56**, 2062–2069.

297 Scheffler, T. L., Scheffler, J. M., Park, S., Kasten, S. C., Wu, Y., McMillan, R. P., Hulver, M. W., Frisard, M. I. and Gerrard, D. E. (2014) Fiber hypertrophy and increased oxidative capacity can occur simultaneously in pig glycolytic skeletal muscle. Am J Physiol Cell Physiol **306**, C354–63.

298 Leick, L., Hellsten, Y., Fentz, J., Lyngby, S. S., Wojtaszewski, J. F. P., Hidalgo, J. and Pilegaard, H. (2009) PGC-1alpha mediates exercise-induced skeletal muscle VEGF expression in mice. Am. J. Physiol. Endocrinol. Metab. **297**, E92–E103.

299 Garcia-Roves, P. M., Osler, M. E., Holmström, M. H. and Zierath, J. R. (2008) Gain-of-function R225Q mutation in AMP-activated protein kinase γ3 subunit increases mitochondrial biogenesis in glycolytic skeletal muscle. J. Biol. Chem. **283**, 35724–35734.

300 O'Neill, H. M., Holloway, G. P. and Steinberg, G. R. (2013) Molecular and Cellular Endocrinology AMPK regulation of fatty acid metabolism and

mitochondrial biogenesis: Implications for obesity. Mol. Cell. Endocrinol. **366**, 135–151.

301 Isola, L. M., Zhou, S. L., Kiang, C. L., Stump, D. D., Bradbury, M. W. and Berk, P. D. (1995) 3T3 fibroblasts transfected with a cDNA for mitochondrial aspartate aminotransferase express plasma membrane fatty acid-binding protein and saturable fatty acid uptake. Proc. Natl. Acad. Sci. U. S. A. **92**, 9866–9870.

302 Schwieterman, W., Sorrentino, D., Potter, B. J., Rand, J., Kiang, C. L., Stump, D. and Berk, P. D. (1988) Uptake of oleate by isolated rat adipocytes is mediated by a 40-kDa plasma membrane fatty acid binding protein closely related to that in liver and gut. Proc. Natl. Acad. Sci. U. S. A. **85**, 359–363.

303 Stremmel, W., Lotz, G., Strohmeyer, G. and Berk, P. D. (1985) Identification, isolation, and partial characterization of a fatty acid binding protein from rat jejunal microvillous membranes. J. Clin. Invest. **75**, 1068–1076.

304 Hirsch, D., Stahl, A. and Lodish, H. F. (1998) A family of fatty acid transporters conserved from mycobacterium to man. Proc. Natl. Acad. Sci. U. S. A. **95**, 8625–8629.

305 Gimeno, R. E., Ortegon, A. M., Patel, S., Punreddy, S., Ge, P., Sun, Y., Lodish, H. F. and Stahl, A. (2003) Characterization of a heart-specific fatty acid transport protein. J. Biol. Chem. **278**, 16039–16044.

306 Abumrad, N. A., El-Maghrabi, M. R., Amri, E. Z., Lopez, E. and Grimaldi, P. A. (1993) Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. J. Biol. Chem. **268**, 17665–17668.

307 Holloway, G. P., Chou, C. J., Lally, J., Stellingwerff, T., Maher, A. C., Gavrilova, O., Haluzik, M., Alkhateeb, H., Reitman, M. L. and Bonen, A. (2011) Increasing skeletal muscle fatty acid transport protein 1 (FATP1) targets fatty acids to oxidation and does not predispose mice to diet-induced insulin resistance. Diabetologia **54**, 1457–1467.

308 García-Martínez, C., Marotta, M., Moore-Carrasco, R., Guitart, M., Camps, M., Busquets, S., Montell, E. and Gómez-Foix, A. M. (2005) Impact on fatty acid metabolism and differential localization of FATP1 and

FAT/CD36 proteins delivered in cultured human muscle cells. Am. J. Physiol. Cell Physiol. **288**, C1264–C1272.

309 Bonen, A., Parolin ML, Steinberg GR, Calles-Escandon J, Tandon NN, Glatz JF, Luiken JJ, Heigenhauser GJ, D. D. (2004) Triacylglycerol accumulation in human obesity and type 2 diabetes is associated with increased rates of skeletal muscle fatty acid transport and increased sarcolemmal FAT/CD36. FASEB J. **18**, 114–6.

310 Bonen, A., Holloway, G. P., Tandon, N. N., Han, X. X., McFarlan, J., Glatz, J. F. and Luiken, J. J. (2009) Cardiac and skeletal muscle fatty acid transport and transporters and triacylglycerol and fatty acid oxidation in lean and Zucker diabetic fatty rats. Am J Physiol Regul Integr Comp Physiol **297**, R1202–12.

311 Han, X.-X., Chabowski, A., Tandon, N. N., Calles-Escandon, J., Glatz, J. F. C., Luiken, J. J. F. P. and Bonen, A. (2007) Metabolic challenges reveal impaired fatty acid metabolism and translocation of FAT/CD36 but not FABPpm in obese Zucker rat muscle. Am. J. Physiol. Endocrinol. Metab. **293**, E566–E575.

312 Holloway, G. P., Benton, C. R., Mullen, K. L., Yoshida, Y., Snook, L. A., Han, X.-X., Glatz, J. F. C., Luiken, J. J. F. P., Lally, J., Dyck, D. J., et al. (2009) In obese rat muscle transport of palmitate is increased and is channeled to triacylglycerol storage despite an increase in mitochondrial palmitate oxidation. Am. J. Physiol. Endocrinol. Metab. **296**, E738–E747.

313 Luiken, J. J., Arumugam, Y., Dyck, D. J., Bell, R. C., Pelsers, M. M., Turcotte, L. P., Tandon, N. N., Glatz, J. F. and Bonen, A. (2001) Increased rates of fatty acid uptake and plasmalemmal fatty acid transporters in obese Zucker rats. J. Biol. Chem. **276**, 40567–40573.

314 Smith, A. C., Mullen, K. L., Junkin, K. A., Nickerson, J., Chabowski, A., Bonen, A. and Dyck, D. J. (2007) Metformin and exercise reduce muscle FAT/CD36 and lipid accumulation and blunt the progression of high-fat diet-induced hyperglycemia. Am. J. Physiol. Endocrinol. Metab. **293**, E172–E181.

315 Aguer, C., Mercier, J., Yong Wai Man, C., Metz, L., Bordenave, S., Lambert, K., Jean, E., Lantier, L., Bounoua, L., Brun, J. F., et al. (2010) Intramyocellular lipid accumulation is associated with permanent relocation ex vivo and in vitro of fatty acid translocase (FAT)/CD36 in obese patients. Diabetologia **53**, 1151–1163.

316 Coort, S. L. M., Hasselbaink, D. M., Koonen, D. P. Y., Willems, J., Coumans, W. A., Chabowski, A., Van Der Vusse, G. J., Bonen, A., Glatz, J. F. C. and Luiken, J. J. F. P. (2004) Enhanced sarcolemmal FAT/CD36 content and triacylglycerol storage in cardiac myocytes from obese Zucker rats. Diabetes **53**, 1655–1663.

317 Boden, G., Cheung, P. and Homko, C. (2003) Effects of acute insulin excess and deficiency on gluconeogenesis and glycogenolysis in type 1 diabetes. Diabetes **52**, 133–137.

318 Fentz, J., Kjøbsted, R., Birk, J., Jordy, A., Jeppesen, J., Thorsen, K., Schjerling, P., Kiens, B., Jessen, N., Viollet, B., et al. (2015) AMPKα is critical for enhancing skeletal muscle fatty acid utilization during in vivo exercise in mice. FASEB J. **29**, 1725–1738.

319 Glatz, J. F., Luiken, J. J. and Bonen, a. (2010) Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. Physiol. Rev. **90**, 367–417.

320 Pepino, M. Y., Kuda, O., Samovski, D. and Abumrad, N. a. (2014) Structure-Function of CD36 and Importance of Fatty Acid Signal Transduction in Fat Metabolism. Annu. Rev. Nutr. **34**, 281–303.

321 Samovski, D., Sun, J., Pietka, T., Gross, R., Eckel, R., Su, X., Stahl, P. and Abumrad, N. (2015) Regulation of AMPK activation by CD36 links fatty acid uptake to β-oxidation. Diabetes **64**, 353–9.

322 Love-Gregory, L. and Abumrad, N. A. (2011) CD36 genetics and the metabolic complications of obesity. Curr. Opin. Clin. Nutr. Metab. Care **14**, 527–534.

323 Jeppesen, J., Albers, P. H., Rose, A. J., Birk, J. B., Schjerling, P., Dzamko, N., Steinberg, G. R. and Kiens, B. (2011) Contraction-induced skeletal muscle FAT/CD36 trafficking and FA uptake is AMPK independent. J. Lipid Res. **52**, 699–711.

324 Abbott, M. J., Edelman, A. M. and Turcotte, L. P. (2009) CaMKK is an upstream signal of AMP-activated protein kinase in regulation of substrate metabolism in contracting skeletal muscle. Am. J. Physiol. Regul. Integr. Comp. Physiol. **297**, R1724–R1732.

325 McGarry, J.D., Mills, S.E., Long, C.S., Foster, D. W. . (1983) Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the

presence of malonyl-CoA in non-hepatic tissues of the rat. Biochem. J. **214**, 21–28.

326 O'Neill, H. M., Lally, J. S., Galic, S., Thomas, M., Azizi, P. D., Fullerton, M. D., Smith, B. K., Pulinilkunnil, T., Chen, Z., Samaan, M. C., et al. (2014) AMPK phosphorylation of ACC2 is required for skeletal muscle fatty acid oxidation and insulin sensitivity in mice. Diabetologia **57**, 1693–1702.

327 Winder, W. W., Wilson, H. A., Hardie, D. G., Rasmussen, B. B., Hutber, C. A., Call, G. B., Clayton, R. D., Conley, L. M., Yoon, S. and Zhou, B. (1997) Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. J. Appl. Physiol. **82**, 219–225.

328 Assifi, M. M., Suchankova, G., Constant, S., Prentki, M., Saha, A. K. and Ruderman, N. B. (2005) AMP-activated protein kinase and coordination of hepatic fatty acid metabolism of starved/carbohydrate-refed rats. Am J Physiol Endocrinol Metab **289**, E794–800.

329 Reszko, A. E., Kasumov, T., David, F., Thomas, K. R., Jobbins, K. A., Cheng, J. F., Lopaschuk, G. D., Dyck, J. R. B., Diaz, M., Des Rosiers, C., et al. (2004) Regulation of malonyl-CoA concentration and turnover in the normal heart. J. Biol. Chem. **279**, 34298–34301.

330 Bradbury, M. W. (2006) Lipid metabolism and liver inflammation. I. Hepatic fatty acid uptake: possible role in steatosis. Am. J. Physiol. Gastrointest. Liver Physiol. **290**, G194–G198.

331 Abu-Elheiga, L., Brinkley, W. R., Zhong, L., Chirala, S. S., Woldegiorgis, G. and Wakil, S. J. (2000) The subcellular localization of acetyl-CoA carboxylase 2. Proc. Natl. Acad. Sci. U. S. A. **97**, 1444–1449.

332 Wakil, S. J. and Abu-Elheiga, L. A. (2009) Fatty acid metabolism: target for metabolic syndrome. J. Lipid Res. **50**, S138–S143.

333 Abu-Elheiga, L., Almarza-Ortega, D. B., Baldini, A. and Wakil, S. J. (1997) Human acetyl-CoA carboxylase 2. Molecular cloning, characterization, chromosomal mapping, and evidence for two isoforms. J. Biol. Chem. **272**, 10669–10677.

334 Abu-Elheiga, L., Matzuk, M. M., Abo-Hashema, K. A. and Wakil, S. J. (2001) Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. Science **291**, 2613–2616.

335 Hoehn, K. L., Turner, N., Swarbrick, M. M., Wilks, D., Preston, E., Phua, Y., Joshi, H., Furler, S. M., Larance, M., Hegarty, B. D., et al. (2010) Acute or Chronic Upregulation of Mitochondrial Fatty Acid Oxidation Has No Net Effect on Whole-Body Energy Expenditure or Adiposity. Cell Metab. **11**, 70–76.

336 Olson, D. P., Pulinilkunnil, T., Cline, G. W., Shulman, G. I. and Lowell, B. B. (2010) Gene knockout of Acc2 has little effect on body weight, fat mass, or food intake. Proc. Natl. Acad. Sci. U. S. A. **107**, 7598–7603.

337 Wolfrum, C., Asilmaz, E., Luca, E., Friedman, J. M. and Stoffel, M. (2004) Foxa2 regulates lipid metabolism and ketogenesis in the liver during fasting and in diabetes. Nature **432**, 1027–1032.

338 Barber, M. C., Price, N. T. and Travers, M. T. (2005) Structure and regulation of acetyl-CoA carboxylase genes of metazoa. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids **1733**, 1–28.

339 Shimano, H., Horton, J. D., Hammer, R. E., Shimomura, I., Brown, M. S. and Goldstein, J. L. (1996) Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. J. Clin. Invest. **98**, 1575–1584.

340 Lopez, J. M., Bennett, M. K., Sanchez, H. B., Rosenfeld, J. M. and Osborne, T. F. (1996) Sterol regulation of acetyl coenzyme A carboxylase: a mechanism for coordinate control of cellular lipid. Proc. Natl. Acad. Sci. U. S. A. **93**, 1049–1053.

341 Bennett, M. K., Lopez, J. M., Sanchez, H. B. and Osborne, T. F. (1995) Sterol regulation of fatty acid synthase promoter: Coordinate feedback regulation of two major lipid pathways. J. Biol. Chem. **270**, 25578–25583.

342 Witters, L. A., Watts, T. D., Daniels, D. L. and Evans, J. L. (1988) Insulin stimulates the dephosphorylation and activation of acetyl-CoA carboxylase. Proc. Natl. Acad. Sci. U. S. A. **85**, 5473–5477.

343 Berglund, E. D., Kang, L., Lee-Young, R. S., Hasenour, C. M., Lustig, D. G., Lynes, S. E., Donahue, E. P., Swift, L. L., Charron, M. J. and Wasserman, D. H. (2010) Glucagon and lipid interactions in the regulation of hepatic AMPK signaling and expression of PPARalpha and FGF21 transcripts in vivo. Am. J. Physiol. Endocrinol. Metab. **299**, E607–E614.

344 Halestrap, A. P. and Denton, R. M. (1974) Hormonal regulation of adipose-tissue acetyl-Coenzyme A carboxylase by changes in the polymeric state of

the enzyme. The role of long-chain fatty acyl-Coenzyme A thioesters and citrate. Biochem. J. **142**, 365–377.

345 Hagopian, K. and Munday, M. R. (1997) The role of pyruvate dehydrogenase, phosphofructo-1-kinase and acetyl-CoA carboxylase in the regulation of fatty acid synthesis in the lactating rat mammary gland during the starved to re-fed transition. Biochim. Biophys. Acta - Gen. Subj. **1336**, 474–484.

346 Davies, S. P., Sim, A. T. and Hardie, D. G. (1990) Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase. Eur. J. Biochem. **187**, 183–190.

347 Ha, J., Daniel, S., Broyles, S. S. and Kim, K. H. (1994) Critical phosphorylation sites for acetyl-CoA carboxylase activity. J. Biol. Chem. **269**, 22162–8.

348 Kim, J. M., Yoon, M., Kang, I., Kim, S. S. and Ha, J. (1998) Evidence that acetyl-CoA carboxylase isoforms play different biological roles in H9c2 cardiomyocyte. Biochem. Biophys. Res. Commun. **248**, 490–496.

349 Davies, S. P., Carling, D., Munday, M. R. and Hardie, D. G. (1992) Diurnal rhythm of phosphorylation of rat liver acetyl-CoA carboxylase by the AMP-activated protein kinase, demonstrated using freeze-clamping. Effects of high fat diets. Eur. J. Biochem. **203**, 615–623.

350 Hardie, D. G., Scott, J. W., Pan, D. A. and Hudson, E. R. (2003) Management of cellular energy by the AMP-activated protein kinase system. FEBS Lett. **546**, 113–120.

351 Munday, M. R., Campbell, D. G., Carling, D. and Hardie, D. G. (1988) Identification by amino acid sequencing of three major regulatory phosphorylation sites on rat acetyl-CoA carboxylase. Eur. J. Biochem. **175**, 331–338.

352 Fullerton, M. D., Galic, S., Marcinko, K., Sikkema, S., Pulinilkunnil, T., Chen, Z.-P., O'Neill, H. M., Ford, R. J., Palanivel, R., O'Brien, M., et al. (2013) Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. Nat. Med. **19**, 1649–1654.

353 Saha, A. K., Schwarsin, A. J., Roduit, R., Masse, F., Kaushik, V., Tornheim, K., Prentki, M. and Ruderman, N. B. (2000) Activation of malonyl-CoA decarboxylase in rat skeletal muscle by contraction and the

AMP-activated protein kinase activator 5-aminoimidazole-4-carboxamide-1-beta -D-ribofuranoside. J. Biol. Chem. **275**, 24279–24283.

354 Jang, S. H., Cheesbrough, T. M. and Kolattukudy, P. E. (1989) Molecular cloning, nucleotide sequence, and tissue distribution of malonyl-CoA decarboxylase. J Biol Chem **264**, 3500–3505.

355 Roepstorff, C., Halberg, N., Hillig, T., Saha, A. K., Ruderman, N. B., Wojtaszewski, J. F. P., Richter, E. A. and Kiens, B. (2005) Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. Am. J. Physiol. Endocrinol. Metab. **288**, E133–E142.

356 Odland, L. M., Howlett, R. A., Heigenhauser, G. J., Hultman, E. and Spriet, L. L. (1998) Skeletal muscle malonyl-CoA content at the onset of exercise at varying power outputs in humans. Am. J. Physiol. **274**, E1080–E1085.

357 Smith, B. K., Perry, C. G. R., Koves, T. R., Wright, D. C., Smith, J. C., Neufer, P. D., Muoio, D. M. and Holloway, G. P. (2012) Identification of a novel malonyl-CoA IC(50) for CPT-I: implications for predicting in vivo fatty acid oxidation rates. Biochem. J. **448**, 13–20.

358 Jeppesen, J., Maarbjerg, S. J., Jordy, A. B., Fritzen, A. M., Pehmøller, C., Sylow, L., Serup, A. K., Jessen, N., Thorsen, K., Prats, C., et al. (2013) LKB1 regulates lipid oxidation during exercise independently of AMPK. Diabetes **62**, 1490–1499.

359 Galic, S., Fullerton, M. D., Schertzer, J. D., Sikkema, S., Marcinko, K., Walkley, C. R., Izon, D., Honeyman, J., Chen, Z. P., Van Denderen, B. J., et al. (2011) Hematopoietic AMPK β1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. J. Clin. Invest. **121**, 4903–4915.

360 Carlson, C. L. and Winder, W. W. (1999) Liver AMP-activated protein kinase and acetyl-CoA carboxylase during and after exercise. J. Appl. Physiol. **86**, 669–674.

361 Camacho, R. C., Donahue, E. P., James, F. D., Berglund, E. D. and Wasserman, D. H. (2005) Energy State of the Liver During Short Term and Exhaustive Exercise in C57BL/6J Mice. Am.J.Physiol Endocrinol.Metab **290**, E405–8.

362 Kawaguchi, T., Osatomi, K., Yamashita, H., Kabashima, T. and Uyeda, K. (2002) Mechanism for fatty acid “sparing” effect on glucose-induced

transcription: Regulation of carbohydrate-responsive element-binding protein by AMP-activated protein kinase. J. Biol. Chem. **277**, 3829–3835.

363 Koo, S. H., Flechner, L., Qi, L., Zhang, X., Screaton, R. A., Jeffries, S., Hedrick, S., Xu, W., Boussouar, F., Brindle, P., et al. (2005) The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature **437**, 1109–1111.

364 Hong, Y. H., Varanasi, U. S., Yang, W. and Leff, T. (2003) AMP-activated protein kinase regulates HNF4alpha transcriptional activity by inhibiting dimer formation and decreasing protein stability. J. Biol. Chem. **278**, 27495–27501.

365 Horike, N., Sakoda, H., Kushiyama, A., Ono, H., Fujishiro, M., Kamata, H., Nishiyama, K., Uchijima, Y., Kurihara, Y., Kurihara, H., et al. (2008) AMP-activated protein kinase activation increases phosphorylation of glycogen synthase kinase 3beta and thereby reduces cAMP-responsive element transcriptional activity and phosphoenolpyruvate carboxykinase C gene expression in the liver. J. Biol. Chem. **283**, 33902–33910.

366 Mashek, D. G. (2013) Hepatic fatty acid trafficking: multiple forks in the road. Adv. Nutr. **4**, 697–710.

367 Falcon, A., Doege, H., Fluitt, A., Tsang, B., Watson, N., Kay, M. A. and Stahl, A. (2010) FATP2 is a hepatic fatty acid transporter and peroxisomal very long-chain acyl-CoA synthetase. Am. J. Physiol. Endocrinol. Metab. **299**, E384–E393.

368 Doege, H., Baillie, R. A., Ortegon, A. M., Tsang, B., Wu, Q., Punreddy, S., Hirsch, D., Watson, N., Gimeno, R. E. and Stahl, A. (2006) Targeted Deletion of FATP5 Reveals Multiple Functions in Liver Metabolism: Alterations in Hepatic Lipid Homeostasis. Gastroenterology **130**, 1245–1258.

369 Rajaraman, G., Roberts, M. S., Hung, D., Wang, G. Q. and Burczynski, F. J. (2005) Membrane binding proteins are the major determinants for the hepatocellular transmembrane flux of long-chain fatty acids bound to albumin. Pharm. Res. **22**, 1793–1804.

370 Stump, D. D., Nunes, R. M., Sorrentino, D., Isola, L. M. and Berk, P. D. (1992) Characteristics of oleate binding to liver plasma membranes and its uptake by isolated hepatocytes. J. Hepatol. **16**, 304–315.

371 Mitsuyoshi, H., Yasui, K., Harano, Y., Endo, M., Tsuji, K., Minami, M., Itoh, Y., Okanoue, T. and Yoshikawa, T. (2009) Analysis of hepatic genes involved in the metabolism of fatty acids and iron in nonalcoholic fatty liver disease. Hepatol. Res. **39**, 366–373.

372 Greco, D., Kotronen, A., Westerbacka, J., Puig, O., Arkkila, P., Kiviluoto, T., Laitinen, S., Kolak, M., Fisher, R. M., Hamsten, A., et al. (2008) Gene expression in human NAFLD. Am. J. Physiol. Gastrointest. Liver Physiol. **294**, G1281–G1287.

373 Bass, N. M. (1990) Fatty acid-binding protein expression in the liver: its regulation and relationship to the zonation of fatty acid metabolism. Mol Cell Biochem **98**, 167–76.

374 Chakravarty, K., Leahy, P., Becard, D., Hakimi, P., Foretz, M., Ferre, P., Foufelle, F. and Hanson, R. W. (2001) Sterol Regulatory Element-binding Protein-1c Mimics the Negative Effect of Insulin on Phosphoenolpyruvate Carboxykinase (GTP) Gene Transcription. J. Biol. Chem. **276**, 34816–34823.

375 Guillet-deniau, I., Mieulet, V., Lay, S. Le, Achouri, Y., Carre, D., Girard, J., Foufelle, F. and Ferre, P. (2002) Sterol Regulatory Element Binding Protein-1c. Diabetes **51**, 1722–1728.

376 Latasa, M. J., Moon, Y. S., Kim, K. H. and Sul, H. S. (2000) Nutritional regulation of the fatty acid synthase promoter in vivo: sterol regulatory element binding protein functions through an upstream region containing a sterol regulatory element. Proc. Natl. Acad. Sci. U. S. A. **97**, 10619–10624.

377 Hegarty, B. D., Bobard, A., Hainault, I., Ferré, P., Bossard, P. and Foufelle, F. (2005) Distinct roles of insulin and liver X receptor in the induction and cleavage of sterol regulatory element-binding protein-1c. Proc. Natl. Acad. Sci. U. S. A. **102**, 791–796.

378 Matsusue, K., Haluzik, M., Lambert, G., Yim, S. H., Gavrilova, O., Ward, J. M., Brewer, B., Reitman, M. L. and Gonzalez, F. J. (2003) Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. J. Clin. Invest. **111**, 737–747.

379 Yu, S., Matsusue, K., Kashireddy, P., Cao, W.-Q., Yeldandi, V., Yeldandi, A. V, Rao, M. S., Gonzalez, F. J. and Reddy, J. K. (2003) Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor gamma1 (PPARgamma1) overexpression. J. Biol. Chem. **278**, 498–505.

380 Shrago, E., Glennon, J. A. and Gordon, E. S. (1971) Comparative aspects of lipogenesis in mammalian tissues. Metabolism. **20**, 54–62.

381 Schwarz, J. M., Linfoot, P., Dare, D. and Aghajanian, K. (2003) Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. Am. J. Clin. Nutr. **77**, 43–50.

382 Parks, E. J., Skokan, L. E., Timlin, M. T. and Dingfelder, C. S. (2008) Dietary sugars stimulate fatty acid synthesis in adults. J. Nutr. **138**, 1039–1046.

383 Aarsland, A. and Wolfe, R. R. (1998) Hepatic secretion of VLDL fatty acids during stimulated lipogenesis in men. J. Lipid Res. **39**, 1280–1286.

384 Paton, C. M. and Ntambi, J. M. (2009) Biochemical and physiological function of stearoyl-CoA desaturase. Am. J. Physiol. Endocrinol. Metab. **297**, E28–E37.

385 Green, C. D., Ozguden-Akkoc, C. G., Wang, Y., Jump, D. B. and Olson, L. K. (2010) Role of fatty acid elongases in determination of de novo synthesized monounsaturated fatty acid species. J. Lipid Res. **51**, 1871–1877.

386 Bjermo, H. and Risérus, U. (2010) Role of hepatic desaturases in obesity-related metabolic disorders. Curr. Opin. Clin. Nutr. Metab. Care **13**, 703–708.

387 Jump, D. B. (2009) Mammalian fatty acid elongases. Methods Mol. Biol. **579**, 375–389.

388 Bu, S. Y. and Mashek, D. G. (2010) Hepatic long-chain acyl-CoA synthetase 5 mediates fatty acid channeling between anabolic and catabolic pathways. J. Lipid Res. **51**, 3270–3280.

389 Bu, S. Y., Mashek, M. T., Mashek, D. G. (2009) Suppression of long chain acyl-CoA synthetase 3 decreases hepatic de novo fatty acid synthesis through decreased transcriptional activity. J Biol Chem **284**, 30474–83.

390 Nagle, C. A., Klett, E. L. and Coleman, R. A. (2009) Hepatic triacylglycerol accumulation and insulin resistance. J. Lipid Res. **50**, S74–S79.

391 Leclerc, I., Lenzner, C., Gourdon, L., Vaulont, S., Kahn, A. and Viollet, B. (2001) Hepatocyte nuclear factor-4alpha involved in type 1 maturity-onset diabetes of the young is a novel target of AMP-activated protein kinase. Diabetes **50**, 1515–1521.

392 Muoio, D. M., Seefeld, K., Witters, L. A. and Coleman, R. A. (1999) AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target. Biochem. J. **338**, 783–791.

393 Paterson, J. M., Morton, N. M., Fievet, C., Kenyon, C. J., Holmes, M. C., Staels, B., Seckl, J. R. and Mullins, J. J. (2004) Metabolic syndrome without obesity: Hepatic overexpression of 11beta-hydroxysteroid dehydrogenase type 1 in transgenic mice. Proc. Natl. Acad. Sci. U. S. A. **101**, 7088–7093.

394 Carling, D., Zammit, V. A. and Hardie, D. G. (1987) A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS Lett. **223**, 217–222.

395 Mao, J., DeMayo, F. J., Li, H., Abu-Elheiga, L., Gu, Z., Shaikenov, T. E., Kordari, P., Chirala, S. S., Heird, W. C. and Wakil, S. J. (2006) Liver-specific deletion of acetyl-CoA carboxylase 1 reduces hepatic triglyceride accumulation without affecting glucose homeostasis. Proc. Natl. Acad. Sci. U. S. A. **103**, 8552–8557.

396 Harada, N., Oda, Z., Hara, Y., Fujinami, K., Okawa, M., Ohbuchi, K., Yonemoto, M., Ikeda, Y., Ohwaki, K., Aragane, K., et al. (2007) Hepatic de novo lipogenesis is present in liver-specific ACC1-deficient mice. Mol. Cell. Biol. **27**, 1881–1888.

397 Savage, D. B., Cheol, S. C., Samuel, V. T., Liu, Z. X., Zhang, D., Wang, A., Zhang, X. M., Cline, G. W., Xing, X. Y., Geisler, J. G., et al. (2006) Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. J. Clin. Invest. **116**, 817–824.

398 Zelber-Sagi, S., Nitzan-Kaluski, D., Goldsmith, R., Webb, M., Zvibel, I., Goldiner, I., Blendis, L., Halpern, Z. and Oren, R. (2008) Role of leisure-time physical activity in nonalcoholic fatty liver disease: a population-based study. Hepatology **48**, 1791–1798.

399 Yoshimura, E., Kumahara, H., Tobina, T., Matsuda, T., Ayabe, M., Kiyonaga, A., Anzai, K., Higaki, Y. and Tanaka, H. (2014) Lifestyle

intervention involving calorie restriction with or without aerobic exercise training improves liver fat in adults with visceral adiposity. J. Obes. **2014**.

400 Liu, W., Lu, D., Du, Y., Sun, J., Ge, J. and Wang, R. et al. (2014) Effect of aerobic exercise and low carbohydrate diet on pre-diabetic non-alcoholic fatty liver disease in postmenopausal women and middle aged men – the role of gut microbiota composition: study protocol for the AELC randomized controlled trial. BMC Public Health **14**.

401 Reinehr, T., Schmidt, C., Toschke, A. M. and Andler, W. (2009) Lifestyle intervention in obese children with non-alcoholic fatty liver disease: 2-year follow-up study. Arch. Dis. Child. **94**, 437–442.

402 Tock, L., Prado, W. L., Caranti, D. A., Cristofalo, D. M. J., Lederman, H., Fisberg, M., Siqueira, K. O., Stella, S. G., Antunes, H. K., Cintra, I. P., et al. (2006) Nonalcoholic fatty liver disease decrease in obese adolescents after multidisciplinary therapy. Eur. J. Gastroenterol. Hepatol. **18**, 1241–1245.

403 Nobili, V., Marcellini, M., Devito, R., Ciampalini, P., Piemonte, F., Comparcola, D., Sartorelli, M. R. and Angulo, P. (2006) NAFLD in children: A prospective clinical-pathological study and effect of lifestyle advice. Hepatology **44**, 458–465.

404 Miyake, T., Kumagi, T., Hirooka, M., Furukawa, S., Kawasaki, K. and Koizumi, M., et al. (2015) Significance of exercise in nonalcoholic fatty liver disease in men: a community-based large cross-sectional study. J Gastroenterol **50**, 230–7.

405 Asad, MR., Haddadi, F., Nejad, MR., Sokhtehzari, S. (2013) Effect of eight weeks endurance exercise on liver enzymes in stopping drug women with methadone. J. Paramed. Sci. **4**, 68–71.

406 Kistler, K. D., Brunt, E. M., Clark, J. M., Diehl, A. M., Sallis, J. F. and Schwimmer, J. B. (2011) Physical activity recommendations, exercise intensity, and histological severity of nonalcoholic fatty liver disease. Am. J. Gastroenterol. **106**, 460–468.

407 Gonçalves, I. O., Passos, E., Rocha-Rodrigues, S., Diogo, C. V., Torrella, J. R., Rizo, D., Viscor, G., Santos-Alves, E., Marques-Aleixo, I., Oliveira, P. J., et al. (2014) Physical exercise prevents and mitigates non-alcoholic steatohepatitis-induced liver mitochondrial structural and bioenergetics impairments. Mitochondrion **15**, 40–51.

408 Rector, R. S., Uptergrove, G. M., Morris, E. M., Borengasser, S. J., Laughlin, M. H., Booth, F. W., Thyfault, J. P. and Ibdah, J. A. (2011) Daily exercise vs. caloric restriction for prevention of nonalcoholic fatty liver disease in the OLETF rat model. Am. J. Physiol. Gastrointest. Liver Physiol. **300**, G874–G883.

409 Bucher, J., Krüsi, M., Zueger, T., Ith, M., Stettler, C., Diem, P., Boesch, C., Kreis, R. and Christ, E. (2014) The effect of a single 2 h bout of aerobic exercise on ectopic lipids in skeletal muscle, liver and the myocardium. Diabetologia **57**, 1001–1005.

410 Gauthier, M.-S., Couturier, K., Latour, J.-G. and Lavoie, J.-M. (2003) Concurrent exercise prevents high-fat-diet-induced macrovesicular hepatic steatosis. J. Appl. Physiol. **94**, 2127–2134.

411 Sheldon, R., Laughlin, M. and Rector, R. (2014) Reduced hepatic eNOS phosphorylation is associated with NAFLD and type 2 diabetes progression and is prevented by daily exercise in hyperphagic OLETF rats. J Appl Physiol **116**, 1156–64.

412 Linden, M. A., Meers, G. M., Ruebel, M. L., Jenkins, N. T., Booth, F. W., Laughlin, M. H., Ibdah, J. a, Thyfault, J. P. and Rector, R. S. (2013) Hepatic steatosis development with four weeks of physical inactivity in previously active, hyperphagic OLETF rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. **304**, R763–71.

413 Cho, J., Lee, I. and Kim, D. et al. (2014) Effect of aerobic exercise training on non-alcoholic fatty liver disease induced by a high fat diet in C57BL/6 mice. J Exerc Nutr. Biochem **18**, 339–46.

414 Sene-Fiorese, M., Duarte, F., Scarmagnani, F., Cheik, N., Manzoni, M., Nonaka, K., Rossi, E., de Oliveira Duarte, A. and Dâmaso, A. (2008) Efficiency of intermittent exercise on adiposity and fatty liver in rats fed with high-fat diet. Obes. (Silver Spring) **16**, 2217–22.

415 Haram, P. M., Kemi, O. J., Lee, S. J., Bendheim, M., Al-Share, Q. Y., Waldum, H. L., Gilligan, L. J., Koch, L. G., Britton, S. L., Najjar, S. M., et al. (2009) Aerobic interval training vs. continuous moderate exercise in the metabolic syndrome of rats artificially selected for low aerobic capacity. Cardiovasc. Res. **81**, 723–732.

416 Thyfault, J. P., Rector, R. S., Uptergrove, G. M., Borengasser, S. J., Morris, E. M., Wei, Y., Laye, M. J., Burant, C. F., Qi, N. R., Ridenhour, S. E., et al. (2009) Rats selectively bred for low aerobic capacity have reduced hepatic

mitochondrial oxidative capacity and susceptibility to hepatic steatosis and injury. J. Physiol. **587**, 1805–1816.

417 Cho, J., Kim, S., Lee, S. and Kang, H. (2014) Effect of Training Intensity on Nonalcoholic Fatty Liver Disease. Med Sci Sport. Exerc **47**, 1624–34.

418 Devries, M. C., Samjoo, I. A., Hamadeh, M. J. and Tarnopolsky, M. A. (2008) Effect of endurance exercise on hepatic lipid content, enzymes, and adiposity in men and women. Obes. (Silver Spring) **16**, 2281–2288.

419 Johnson, N. A., Sachinwalla, T., Walton, D. W., Smith, K., Armstrong, A., Thompson, M. W. and George, J. (2009) Aerobic exercise training reduces hepatic and visceral lipids in obese individuals without weight loss. Hepatology **50**, 1105–1112.

420 Tilg, H. and Moschen, A. (2010) Weight loss: cornerstone in the treatment of non-alcoholic fatty liver disease. Minerva Gastroenterol. Dietol. **56**, 159–167.

421 Sarvas, J., Otis, J., Khaper, N. and Lees, S. (2015) Voluntary physical activity prevents insulin resistance in a tissue specific manner. Physiol rep **3**, e12277.

422 Jordy, A. B., Kraakman, M. J., Gardner, T., Estevez, E., Kammoun, H. L., Weir, J. M., Kiens, B., Meikle, P. J., Febbraio, M. A. and Henstridge, D. C. (2015) Analysis of the liver lipidome reveals insights into the protective effect of exercise on high-fat diet-induced hepatosteatosis in mice. Am J Physiol Endocrinol Metab **308**, E778–E791.

423 Rector, R. S., Thyfault, J. P., Morris, R. T., Laye, M. J., Borengasser, S. J., Booth, F. W. and Ibdah, J. A. (2008) Daily exercise increases hepatic fatty acid oxidation and prevents steatosis in Otsuka Long-Evans Tokushima Fatty rats. Am. J. Physiol. Gastrointest. Liver Physiol. **294**, G619–G626.

424 Rector, R. S., Thyfault, J. P., Laye, M. J., Morris, R. T., Borengasser, S. J., Uptergrove, G. M., Chakravarthy, M. V, Booth, F. W. and Ibdah, J. A. (2008) Cessation of daily exercise dramatically alters precursors of hepatic steatosis in Otsuka Long-Evans Tokushima Fatty (OLETF) rats. J. Physiol. **586**, 4241–4249.

425 Johnson, N. A. and George, J. (2010) Fitness versus fatness: Moving beyond weight loss in nonalcoholic fatty liver disease. Hepatology **52**, 370–381.

426 Berglund, E. D., Lustig, D. G., Baheza, R. A., Hasenour, C. M., Lee-Young, R. S., Donahue, E. P., Lynes, S. E., Swift, L. L., Charron, M. J., Damon, B. M., et al. (2011) Hepatic glucagon action is essential for exercise-induced reversal of mouse fatty liver. Diabetes **60**, 2720–2729.

427 Fisher, J. S., Gao, J., Han, D.-H., Holloszy, J. O. and Nolte, L. A. (2002) Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. Am. J. Physiol. Endocrinol. Metab. **282**, E18–E23.

428 Hegarty, B. D., Turner, N., Cooney, G. J. and Kraegen, E. W. (2009) Insulin resistance and fuel homeostasis: The role of AMP-activated protein kinase. Acta Physiol. **196**, 129–145.

429 Viollet, B., Horman, S., Leclerc, J., Lantier, L., Foretz, M., Billaud, M., Giri, S. and Andreelli, F. (2010) AMPK inhibition in health and disease. Crit. Rev. Biochem. Mol. Biol. **45**, 276–295.

430 Lochhead, P. A., Salt, I. P., Walker, K. S., Hardie, D. G. and Sutherland, C. (2000) 5-Aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase. Diabetes **49**, 896–903.

431 Shaw, R. J., Lamia, K. A., Vasquez, D., Koo, S.-H., Bardeesy, N., Depinho, R. A., Montminy, M. and Cantley, L. C. (2005) The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science **310**, 1642–1646.

432 Andreelli, F., Foretz, M., Knauf, C., Cani, P. D., Perrin, C., Iglesias, M. A., Pillot, B., Bado, A., Tronche, F., Mithieux, G., et al. (2006) Liver adenosine monophosphate-activated kinase-α2 catalytic subunit is a key target for the control of hepatic glucose production by adiponectin and leptin but not insulin. Endocrinology **147**, 2432–2441.

433 Hoene, M., Lehmann R, Hennige AM, Pohl AK, Häring HU, Schleicher ED, W. C. (2009) Acute regulation of metabolic genes and insulin receptor substrates in the liver of mice by one single bout of treadmill exercise. J Physiol **587**, 241–52.

434 Kelly, M., Keller, C., Avilucea, P. R., Keller, P., Luo, Z., Xiang, X., Giralt, M., Hidalgo, J., Saha, A. K., Pedersen, B. K., et al. (2004) AMPK activity is diminished in tissues of IL-6 knockout mice: The effect of exercise. Biochem. Biophys. Res. Commun. **320**, 449–454.

435 Graham, G. G., Punt, J., Arora, M., Day, R. O., Doogue, M. P., Duong, J. K., Furlong, T. J., Greenfield, J. R., Greenup, L. C., Kirkpatrick, C. M., et al. (2011) Clinical pharmacokinetics of metformin. Clin. Pharmacokinet. **50**, 81–98.

436 Zheng, J., Woo, S., Hu, X., Botchlett, R., Chen, L., Huo, Y. and Wu, C. (2015) Metformin and metabolic diseases: a focus on hepatic aspects. Front Med **9**, 173–186.

437 Perriello, G., Misericordia, P., Volpi, E., Santucci, A., Santucci, C., Ferrannini, E., Ventura, M. M., Santeusanio, F., Brunetti, P. and Bolli, G. B. (1994) Acute antihyperglycemic mechanisms of metformin in NIDDM: Evidence for suppression of lipid oxidation and hepatic glucose production. Diabetes **43**, 920–928.

438 Viollet, B., Guigas, B., Leclerc, J., Hebrard, S., Lantier, L., Mounier, R., Andreelli, F. and Foretz, M. (2009) AMP-activated protein kinase in the regulation of hepatic energy metabolism: from physiology to therapeutic perspectives. Acta Physiol **196**, 81–98.

439 Viollet, B., Guigas, B., Sanz Garcia, N., Leclerc, J., Foretz, M. and Andreelli, F. (2011) Cellular and molecular mechanisms of metformin: an overview. Clin. Sci. **122**, 253–270.

440 Lin, H. Z., Yang, S. Q., Chuckaree, C., Kuhajda, F., Ronnet, G. and Diehl, A. M. (2000) Metformin reverses fatty liver disease in obese, leptin-deficient mice. Nat. Med. **6**, 998–1003.

441 Foretz, M., Hébrard, S., Leclerc, J., Zarrinpashneh, E., Soty, M., Mithieux, G., Sakamoto, K., Andreelli, F. and Viollet, B. (2010) Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. J. Clin. Invest. **120**, 2355–2369.

442 Miller, R. A., Chu, Q., Xie, J., Foretz, M., Viollet, B. and Birnbaum, M. J. (2013) Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. Nature **494**, 256–60.

443 Thiebaud, D., Jacot, E., DeFronzo, R. A., Maeder, E., Jequier, E. and Felber, J. P. (1982) The effect of graded doses of insulin on total glucose uptake, glucose oxidation, and glucose storage in man. Diabetes **31**, 957–963.

444 Ruderman, N. B., Park, H., Kaushik, V. K., Dean, D., Constant, S., Prentki, M. and Saha, A. K. (2003) AMPK as a metabolic switch in rat muscle, liver and adipose tissue after exercise. Acta Physiol. Scand. **178**, 435–442.

445 Bujak, A. L., Blümer, R. M. E., Marcinko, K., Fullerton, M. D., Kemp, B. E. and Steinberg, G. R. (2014) Reduced skeletal muscle AMPK and mitochondrial markers do not promote age-induced insulin resistance. J. Appl. Physiol. **117**, 171–179.

446 Beck Jørgensen, S., O'Neill, H. M., Hewitt, K., Kemp, B. E. and Steinberg, G. R. (2009) Reduced AMP-activated protein kinase activity in mouse skeletal muscle does not exacerbate the development of insulin resistance with obesity. Diabetologia **52**, 2395–2404.

447 Frøsig, C., Jensen, T. E., Jeppesen, J., Pehmøller, C., Treebak, J. T., Maarbjerg, S. J., Kristensen, J. M., Sylow, L., Alsted, T. J., Schjerling, P., et al. (2013) AMPK and insulin action--responses to ageing and high fat diet. PLoS One **8**, e62338.

448 Goodyear, L. J. (2008) The exercise pill--too good to be true? N. Engl. J. Med. **359**, 1842–1844.

449 Liu, Y., Wan, Q., Guan, Q., Gao, L. and Zhao, J. (2006) High-fat diet feeding impairs both the expression and activity of AMPKa in rats' skeletal muscle. Biochem. Biophys. Res. Commun. **339**, 701–707.

450 Martin, T. L., Alquier, T., Asakura, K., Furukawa, N., Preitner, F. and Kahn, B. B. (2006) Diet-induced obesity alters AMP kinase activity in hypothalamus and skeletal muscle. J. Biol. Chem. **281**, 18933–18941.

451 Fujii, N., Ho, R. C., Manabe, Y., Jessen, N., Toyoda, T., Holland, W. L., Summers, S. A., Hirshman, M. F. and Goodyear, L. J. (2008) Ablation of AMP-activated protein kinase α2 activity exacerbates insulin resistance induced by high-fat feeding of mice. Diabetes **57**, 2958–2966.

452 Gomes, A. P., Duarte, F. V., Nunes, P., Hubbard, B. P., Teodoro, J. S., Varela, A. T., Jones, J. G., Sinclair, D. A., Palmeira, C. M. and Rolo, A. P. (2012) Berberine protects against high fat diet-induced dysfunction in muscle mitochondria by inducing SIRT1-dependent mitochondrial biogenesis. Biochim. Biophys. Acta - Mol. Basis Dis. **1822**, 185–195.

453 Al-Khalili, L., Forsgren, M., Kannisto, K., Zierath, J. R., Lönnqvist, F. and Krook, A. (2005) Enhanced insulin-stimulated glycogen synthesis in response to insulin, metformin or rosiglitazone is associated with increased

mRNA expression of GLUT4 and peroxisomal proliferator activator receptor gamma co-activator 1. Diabetologia **48**, 1173–1179.

454 Chen, Y. C., Lee, S. Da, Ho, L. T. and Kuo, C. H. (2011) The Effects of Altitude Training on the AMPK-Related Glucose Transport Pathway in the Red Skeletal Muscle of Both Lean and Obese Zucker Rats. High Alt. Med. Biol. **12**, 371–378.

455 Ivy, J. L., Sherman, W. M., Cutler, C. L. and Katz, A. L. (1986) Exercise and diet reduce muscle insulin resistance in obese Zucker rat. Am. J. Physiol. **251**, E299–E305.

456 Kraegen, E. W., Storlien, L. H., Jenkins, A. B. and James, D. E. (1989) Chronic exercise compensates for insulin resistance induced by a high-fat diet in rats. Am. J. Physiol. **256**, E242–E249.

457 Hamada, T., Arias, E. B. and Cartee, G. D. (2006) Increased submaximal insulin-stimulated glucose uptake in mouse skeletal muscle after treadmill exercise. J. Appl. Physiol. **101**, 1368–1376.

458 Jessen, N., Pold, R., Buhl, E. S., Jensen, L. S., Schmitz, O. and Lund, S. (2003) Effects of AICAR and exercise on insulin-stimulated glucose uptake, signaling, and GLUT-4 content in rat muscles. J. Appl. Physiol. **94**, 1373–1379.

459 Koshinaka, K., Kawasaki, E., Hokari, F. and Kawanaka, K. (2009) Effect of acute high-intensity intermittent swimming on post-exercise insulin responsiveness in epitrochlearis muscle of fed rats. Metabolism. **58**, 246–253.

460 Sharoff, C. G., Hagobian, T. A., Malin, S. K., Chipkin, S. R., Yu, H., Hirshman, M. F., Goodyear, L. J. and Braun, B. (2010) Combining short-term metformin treatment and one bout of exercise does not increase insulin action in insulin-resistant individuals. Am. J. Physiol. Endocrinol. Metab. **298**, E815–E823.

461 Wojtaszewski, J. F., Hansen, B. F., Gade, Kiens, B., Markuns, J. F., Goodyear, L. J. and Richter, E. A. (2000) Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. Diabetes **49**, 325–331.

462 Frøsig, C., Jørgensen, S. B., Hardie, D. G., Richter, E. A. and Wojtaszewski, J. F. P. (2004) 5'-AMP-activated protein kinase activity and protein expression are regulated by endurance training in human skeletal muscle. Am. J. Physiol. Endocrinol. Metab. **286**, E411–E417.

463 Layne, A. S., Nasrallah, S., South, M. A., Howell, M. E. A., McCurry, M. P., Ramsey, M. W., Stone, M. H. and Stuart, C. A. (2011) Impaired muscle AMPK activation in the metabolic syndrome may attenuate improved insulin action after exercise training. J. Clin. Endocrinol. Metab. **96**, 1815–1826.

464 Vind, B. F., Pehmøller, C., Treebak, J. T., Birk, J. B., Hey-Mogensen, M., Beck-Nielsen, H., Zierath, J. R., Wojtaszewski, J. F. P. and Højlund, K. (2011) Impaired insulin-induced site-specific phosphorylation of TBC1 domain family, member 4 (TBC1D4) in skeletal muscle of type 2 diabetes patients is restored by endurance exercise-training. Diabetologia **54**, 157–167.

465 Kim, J., Solis, R. S., Arias, E. B. and Cartee, G. D. (2004) Postcontraction insulin sensitivity: relationship with contraction protocol, glycogen concentration, and 5' AMP-activated protein kinase phosphorylation. J. Appl. Physiol. **96**, 575–583.

466 Abbott, M. Turcotte, L. (2014) AMPK-α2 is involved in exercise training-induced adaptations in insulin-stimulated metabolism in skeletal muscle following high-fat diet. J. Appl. Physiol. **117**, 869–879.

467 Kjøbsted, R., Treebak, J., Fentz, J., Lantier, L., Viollet, B., Birk, J., Schjerling, P., Björnholm, M., Zierath, J. and Wojtaszewski, J. (2015) Prior AICAR stimulation increases insulin sensitivity in mouse skeletal muscle in an AMPK-dependent manner. Diabetes **64**, 2042–2055.

468 Yang, Z., Wang, X., He, Y., Qi, L., Yu, L., Xue, B. and Shi, H. (2012) The Full Capacity of AICAR to Reduce Obesity-Induced Inflammation and Insulin Resistance Requires Myeloid SIRT1. PLoS One **7**, e49935.

469 Lee-Young, R. S., Bonner, J. S., Mayes, W. H., Iwueke, I., Barrick, B. A., Hasenour, C. M., Kang, L. and Wasserman, D. H. (2013) AMP-activated protein kinase (AMPK)α2 plays a role in determining the cellular fate of glucose in insulin-resistant mouse skeletal muscle. Diabetologia **56**, 608–617.

470 Chen, L. L., Zhang, H. H., Zheng, J., Hu, X., Kong, W., Hu, D., Wang, S. X. and Zhang, P. (2011) Resveratrol attenuates high-fat diet-induced insulin resistance by influencing skeletal muscle lipid transport and subsarcolemmal mitochondrial β-oxidation. Metabolism. **60**, 1598–1609.

471 Kumar, N. and Dey, C. S. (2002) Metformin enhances insulin signalling in insulin-dependent and-independent pathways in insulin resistant muscle cells. Br. J. Pharmacol. **137**, 329–336.

472 Kristensen, J. M., Treebak, J. T., Schjerling, P., Goodyear, L. and Wojtaszewski, J. F. P. (2014) Two weeks of metformin treatment induces AMPK-dependent enhancement of insulin-stimulated glucose uptake in mouse soleus muscle. Am. J. Physiol. Endocrinol. Metab. **306**, E1099–109.

473 Musi, N., Hirshman, M. F., Nygren, J., Svanfeldt, M., Bavenholm, P., Rooyackers, O., Zhou, G., Williamson, J. M., Ljunqvist, O., Efendic, S., et al. (2002) Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. Diabetes **51**, 2074–2081.

474 Natali, A., Natali, A., Ferrannini, E. and Ferrannini, E. (2006) Effects of metformin and thiazolidinediones on suppression of hepatic glucose production and stimulation of glucose uptake in type 2 diabetes: a systematic review. Diabetologia **49**, 434–41.

475 Jenkins, Y., Sun, T. Q., Markovtsov, V., Foretz, M., Li, W., Nguyen, H., Li, Y., Pan, A., Uy, G., Gross, L., et al. (2013) AMPK activation through mitochondrial regulation results in increased substrate oxidation and improved metabolic parameters in models of diabetes. PLoS One **8**.

476 Wilcock, C. and Bailey, C. J. (1994) Accumulation of metformin by tissues of the normal and diabetic mouse. Xenobiotica. **24**, 49–57.

477 Cao, J., Meng, S., Chang, E., Beckwith-Fickas, K., Xiong, L., Cole, R. N., Radovick, S., Wondisford, F. E. and He, L. (2014) Low concentrations of metformin suppress glucose production in hepatocytes through AMP-activated protein kinase (AMPK). J. Biol. Chem. **289**, 20435–20446.

478 He, L. and Wondisford, F. E. (2015) Metformin Action: Concentrations Matter. Cell Metab. **21**, 159–162.

479 Madiraju, A. K., Erion, D. M., Rahimi, Y., Zhang, X.-M., Braddock, D. T., Albright, R. a, Prigaro, B. J., Wood, J. L., Bhanot, S., MacDonald, M. J., et al. (2014) Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. Nature **510**, 542–6.

480 Lee, Y. J., Jeschke, G. R., Roelants, F. M., Thorner, J. and Turk, B. E. (2012) Reciprocal Phosphorylation of Yeast Glycerol-3-Phosphate Dehydrogenases in Adaptation to Distinct Types of Stress. Mol. Cell. Biol. **32**, 4705–4717.

481 Bailey, C. and Turner, R. (1996) Metformin. N. Engl. J. Med. **334**, 574–579.

482 Stocker, S. L., Morrissey, K. M., Yee, S. W., Castro, R. A., Xu, L., Dahlin, A., Ramirez, A. H., Roden, D. M., Wilke, R. A., McCarty, C. A., et al. (2013) The effect of novel promoter variants in MATE1 and MATE2 on the pharmacokinetics and pharmacodynamics of metformin. Clin. Pharmacol. Ther. **93**, 186–94.

483 Tucker, G. T., Casey, C., Phillips, P. J., Connor, H., Ward, J. D. and Woods, H. F. (1981) Metformin kinetics in healthy subjects and in patients with diabetes mellitus. Br. J. Clin. Pharmacol. **12**, 235–246.

484 Meng, S., Cao, J., Qiyi, H., Lishou, X., Chang, E., Radovick, S., Wondisford, F. and He, L. (2015) Metformin Activates AMP-activated Protein Kinase by Promoting Formation of the αβγ Heterotrimeric Complex. J. Biol. Chem. **290**, 3793–802.

485 Jablonski, K. A., McAteer, J. B., De Bakker, P. I. W., Franks, P. W., Pollin, T. I., Hanson, R. L., Saxena, R., Fowler, S., Shuldiner, A. R., Knowler, W. C., et al. (2010) Common variants in 40 genes assessed for diabetes incidence and response to metformin and lifestyle intervention in the diabetes prevention program. Diabetes **59**, 2672–2681.

486 Martin-Montalvo, A., Mercken, E. M., Mitchell, S. J., Palacios, H. H., Mote, P. L., Scheibye-Knudsen, M., Gomes, A. P., Ward, T. M., Minor, R. K., Blouin, M. J., et al. (2013) Metformin improves healthspan and lifespan in mice. Nat. Commun. **4**, 2192.

487 Larsen, S., Rabøl, R., Hansen, C. N., Madsbad, S., Helge, J. W. and Dela, F. (2012) Metformin-treated patients with type 2 diabetes have normal mitochondrial complex i respiration. Diabetologia **55**, 443–449.

488 Duca, F., Côté, C., Rasmussen, B., Zadeh-Tahmasebi, M., Rutter, G., Filippi, B. and Lam, T. (2015) Metformin activates a duodenal Ampk-dependent pathway to lower hepatic glucose production in rats. Nat. Med. **21**, 506–511.

489 Maida, A., Lamont, B. J., Cao, X. and Drucker, D. J. (2011) Metformin regulates the incretin receptor axis via a pathway dependent on peroxisome proliferator-activated receptor-α in mice. Diabetologia **54**, 339–349.

490 Wang, P. Y. T., Caspi, L., Lam, C. K. L., Chari, M., Li, X., Light, P. E., Gutierrez-Juarez, R., Ang, M., Schwartz, G. J. and Lam, T. K. T. (2008)

Upper intestinal lipids trigger a gut-brain-liver axis to regulate glucose production. Nature **452**, 1012–1016.

491 Yusta, B., Baggio, L. L., Estall, J. L., Koehler, J. A., Holland, D. P., Li, H., Pipeleers, D., Ling, Z. and Drucker, D. J. (2006) GLP-1 receptor activation improves beta cell function and survival following induction of endoplasmic reticulum stress. Cell Metab. **4**, 391–406.

492 An, J., Muoio, D. M., Shiota, M., Fujimoto, Y., Cline, G. W., Shulman, G. I., Koves, T. R., Stevens, R., Millington, D. and Newgard, C. B. (2004) Hepatic expression of malonyl-CoA decarboxylase reverses muscle, liver and whole-animal insulin resistance. Nat. Med. **10**, 268–274.

493 Liu, X., Zhang, J., Ming, Y., Chen, X., Zeng, M. and Mao, Y. (2015) The aggravation of mitochondrial dysfunction in nonalcoholic fatty liver disease accompanied with type 2 diabetes mellitus. Scand J Gastroenterol **50**, 1152–9.

494 Gonçalves, I. O., Oliveira, P. J., Ascensão, A. and Magalhães, J. (2013) Exercise as a therapeutic tool to prevent mitochondrial degeneration in nonalcoholic steatohepatitis. Eur. J. Clin. Invest. **43**, 1184–1194.

495 Gonçalves, I. O., Maciel, E., Passos, E., Torrella, J. R., Rizo, D., Viscor, G., Rocha-Rodrigues, S., Santos-Alves, E., Domingues, M. R., Oliveira, P. J., et al. (2014) Exercise alters liver mitochondria phospholipidomic profile and mitochondrial activity in non-alcoholic steatohepatitis. Int. J. Biochem. Cell Biol. **54**, 163–173.

496 Koliaki, C., Szendroedi, J., Kaul, K., Jelenik, T., Nowotny, P., Jankowiak, F., Herder, C., Carstensen, M., Krausch, M., Knoefel, W. T., et al. (2015) Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. Cell Metab. **21**, 739–46.

497 Hinkle, P. C. and Yu, M. L. (1979) The phosphorus/oxygen ratio of mitochondrial oxidative phosphorylation. J. Biol. Chem. **254**, 2450–2455.

498 Khamzina, L., Veilleux, A., Bergeron, S. and Marette, A. (2005) Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: Possible involvement in obesity-linked insulin resistance. Endocrinology **146**, 1473–1481.

499 Tremblay, F., Gagnon, A., Veilleux, A., Sorisky, A. and Marette, A. (2005) Activation of the mammalian target of rapamycin pathway acutely inhibits

insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology **146**, 1328–1337.

500 Um, S. H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P. R., Kozma, S. C., Auwerx, J., et al. (2004) Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature **431**, 200–205.

501 Harrington, L. S., Findlay, G. M. and Lamb, R. F. (2005) Restraining PI3K: mTOR signalling goes back to the membrane. Trends Biochem. Sci. **30**, 35–42.

502 Radimerski, T., Montagne, J., Hemmings-Mieszczak, M. and Thomas, G. (2002) Lethality of Drosophila lacking TSC tumor suppressor function rescued by reducing ds6K signaling. Genes Dev. **16**, 2627–2632.

503 Bae, E. J., Xu, J., Oh, D. Y., Bandyopadhyay, G., Lagakos, W. S., Keshwani, M. and Olefsky, J. M. (2012) Liver-specific p70 S6 kinase depletion protects against hepatic steatosis and systemic insulin resistance. J. Biol. Chem. **287**, 18769–18780.

504 Bolster, D. R., Crozier, S. J., Kimball, S. R. and Jefferson, L. S. (2002) AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. J. Biol. Chem. **277**, 23977–23980.

505 Drake, J. C., Alway, S. E., Hollander, J. M. and Williamson, D. L. (2010) AICAR treatment for 14 days normalizes obesity-induced dysregulation of TORC1 signaling and translational capacity in fasted skeletal muscle. Am. J. Physiol. Regul. Integr. Comp. Physiol. **299**, R1546–R1554.

506 Gautsch, T. A., Anthony, J. C., Kimball, S. R., Paul, G. L., Layman, D. K. and Jefferson, L. S. (1998) Availability of eIF4E regulates skeletal muscle protein synthesis during recovery from exercise. Am. J. Physiol. **274**, C406–C414.

507 Williamson, D. L., Bolster, D. R., Kimball, S. R. and Jefferson, L. S. (2006) Time course changes in signaling pathways and protein synthesis in C2C12 myotubes following AMPK activation by AICAR. Am. J. Physiol. Endocrinol. Metab. **291**, E80–E89.

508 Williamson, D. L., Kubica, N., Kimball, S. R. and Jefferson, L. S. (2006) Exercise-induced alterations in extracellular signal-regulated kinase 1/2 and mammalian target of rapamycin (mTOR) signalling to regulatory

mechanisms of mRNA translation in mouse muscle. J. Physiol. **573**, 497–510.

509 Krause, U., Bertrand, L. and Hue, L. (2002) Control of p70 ribosomal protein S6 kinase and acetyl-CoA carboxylase by AMP-activated protein kinase and protein phosphatases in isolated hepatocytes. Eur J Biochem **269**, 3751–3759.

510 Reiter, A. K., Bolster, D. R., Crozier, S. J., Kimball, S. R. and Jefferson, L. S. (2005) Repression of protein synthesis and mTOR signaling in rat liver mediated by the AMPK activator aminoimidazole carboxamide ribonucleoside. Am. J. Physiol. Endocrinol. Metab. **288**, E980–E988.

511 Fillmore, N., Jacobs, D. L., Mills, D. B., Winder, W. W. and Hancock, C. R. (2010) Chronic AMP-activated protein kinase activation and a high-fat diet have an additive effect on mitochondria in rat skeletal muscle. J. Appl. Physiol. **109**, 511–520.

512 Xu, Q. and Si, L.-Y. (2010) Protective effects of AMP-activated protein kinase in the cardiovascular system. J. Cell. Mol. Med. **14**, 2604–2613.

513 Narkar, V. A., Downes, M., Yu, R. T., Embler, E., Wang, Y. X., Banayo, E., Mihaylova, M. M., Nelson, M. C., Zou, Y., Juguilon, H., et al. (2008) AMPK and PPARdelta Agonists Are Exercise Mimetics. Cell **134**, 405–415.

514 Suga, T., Kinugawa, S., Takada, S., Kadoguchi, T., Fukushima, A., Homma, T., Masaki, Y., Furihata, T., Takahashi, M., Sobirin, M. A., et al. (2014) Combination of exercise training and diet restriction normalizes limited exercise capacity and impaired skeletal muscle function in diet-induced diabetic mice. Endocrinology **155**, 68–80.

Appendix A: Staging of NAFLD (from Angulo 2002 [20]).

TABLE 2. GRADING AND STAGING THE HISTOPATHOLOGICAL LESIONS OF NONALCOHOLIC FATTY LIVER DISEASE.*

Grading for steatosis

Grade 1: <33% of hepatocytes affected
Grade 2: 33% to 66% of hepatocytes affected
Grade 3: >66% of hepatocytes affected

Grading for steatohepatitis

Grade 1, mild
- Steatosis: predominantly macrovesicular, involves up to 66% of lobules
- Ballooning: occasionally observed; zone 3 hepatocytes
- Lobular inflammation: scattered and mild acute inflammation (polymorphonuclear cells) and occasional chronic inflammation (mononuclear cells)
- Portal inflammation: none or mild

Grade 2, moderate
- Steatosis: any degree; usually mixed macrovesicular and microvesicular
- Ballooning: obvious and present in zone 3
- Lobular inflammation: polymorphonuclear cells may be noted in association with ballooned hepatocytes; pericellular fibrosis; mild chronic inflammation may be seen
- Portal inflammation: mild to moderate

Grade 3, severe
- Steatosis: typically involves >66% of lobules (panacinar); commonly mixed steatosis
- Ballooning: predominantly zone 3; marked
- Lobular inflammation: scattered acute and chronic inflammation; polymorphonuclear cells may be concentrated in zone 3 areas of ballooning and perisinusoidal fibrosis
- Portal inflammation: mild to moderate

Staging for fibrosis

Stage 1: zone 3 perivenular, perisinusoidal, or pericellular fibrosis; focal or extensive
Stage 2: as above, with focal or extensive periportal fibrosis
Stage 3: bridging fibrosis, focal or extensive
Stage 4: cirrhosis

*Adapted from Brunt et al.[41] with the permission of the publisher.