Regulation of metabolism and inflammation in liver and skeletal muscle

Annika Nerstedt

UNIVERSITY OF GOTHENBURG

The Lundberg Laboratory for Diabetes Research
Department for Molecular and Clinical Medicine
Institute of Medicine at Sahlgrenska Academy
University of Gothenburg, Sweden

2013

TILL MIN FAMILJ

ABSTRACT

Type 2 diabetes (T2D) is a complex metabolic disorder characterised by hyperinsulinaemia, hyperglycaemia and dyslipidaemia. Obesity is the major risk factor for development of insulin resistance, a main predictor of T2D. Recent evidence indicates that nutrient excess and obesity lead to chronic low-grade inflammation in metabolic tissues, which further promotes insulin resistance.

AMP-activated protein kinase (AMPK), a central regulator of energy homeostasis, increases insulin sensitivity in liver and skeletal muscle and lowers the plasma glucose level, thus reverting the major metabolic disturbances in T2D. Serine/threonine protein kinase 25 (STK25) was found to be differentially expressed in skeletal muscle, comparing AMPKγ3 (*Prkag3*$^{-/-}$) knockout mice to wild-type littermates, indicating a potential role for STK25 in regulation of energy homeostasis in skeletal muscle.

In *Paper I*, genes regulating the circadian rhythm (*Cry2*, *Nr1d1* and *Bhlhb2*) were shown to be differentially expressed in skeletal muscle from wild-type mice treated with the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), while they remained unaltered in AMPKγ3 knockout mice. Furthermore, the respiratory exchange ratio (RER) was elevated during the dark period of observation in wild-type mice reflecting a diurnal shift in substrate utilisation from lipid oxidation at daytime to carbohydrate utilisation during nighttime. However, no day/night shift in the RER profile was observed in *Prkag3*$^{-/-}$ littermates. Thus, this study suggests that APMK, as a central energy sensor, could be one important node linking energy metabolism to the circadian clock function.

In *Papers II and III*, the AMPK agonists, AICAR and metformin, are shown to markedly decrease the expression of IL-6-induced serum amyloid A (*SAA*) cluster genes, haptoglobin and suppressor of cytokine signalling 3 (*SOCS3*) in the human hepatocyte cell line HepG2. By repressing AMPK activity with small interfering (si)RNA the inhibitory effect of AMPK on *SAA* expression by both AICAR and metformin was reversed (*Paper II*), indicating that the effect of the agonists is mediated by AMPK activation. Further, we show that AMPK interferes with IL-6 signalling by decreasing IL-6-induced phosphorylation of Janus kinase 1 (JAK1), src homology 2 domain containing protein tyrosine phosphatase 2 (SHP2) and signal transducer and activator of transcription 3 (STAT3) in HepG2 cells (*Papers II* and *III*). In addition, pharmacological activation of AMPK was shown to repress IL-6-induced inflammation *in vivo* by suppression of STAT3 activity in mouse liver (*Paper III*). This suggests that AMPK is an important intracellular link between metabolic and inflammatory pathways in liver.

In *Paper IV* we show that partial reduction of STK25 by siRNA increases uncoupling protein 3 (UCP3), glucose transporter 1 (GLUT1*),* GLUT4 and hexokinase 2 (HK2*)* in

the rodent myoblast cell line L6, both at mRNA and protein level. Correspondingly, the rates of palmitate oxidation and insulin-stimulated glucose uptake were elevated after partial depletion of STK25. In conclusion, our studies suggest a role of STK25 as a negative regulator of glucose and lipid metabolism in skeletal muscle. Impaired glucose uptake and fatty acid metabolism by skeletal muscle is a hallmark of insulin resistance, and therefore, *S*TK25 could be an important new mediator to be evaluated for therapeutic intervention in T2D and related complications.

Keywords: AMPK; IL-6; Inflammation; Liver; JAK1; STK25; Glucose metabolism; Lipid oxidation; Skeletal muscle; Circadian clock; Type 2 diabetes

ISBN 978-91-628-8562-5
http://hdl.handle.net/2077/31713 Gothenburg 2013

LIST OF PAPERS

The thesis is based on the following papers that will be referred to their roman numerals:

I. Vieira E, Nilsson EC, **Nerstedt A**, Ormestad M, Long YC, Garcia-Roves PM, Zierath JR, Mahlapuu M. *Relationship between AMPK and the transcriptional balance of clock-related genes in skeletal muscle.* Am J Physiol Endocrinol Metab. 2008 Nov;295(5):E1032-7.

II. **Nerstedt A**, Johansson A, Andersson CX, Cansby E, Smith U, Mahlapuu M. *AMP-activated protein kinase inhibits IL-6-stimulated inflammatory response in human liver cells by suppressing phosphorylation of signal transducer and activator of transcription 3 (STAT3).* Diabetologia. 2010 Nov;53(11):2406-16.

III. **Nerstedt A,** Cansby E, Amrutkar M, Smith U, Mahlapuu M. *Pharmacological activation of AMPK suppresses inflammatory response evoked by IL-6 signaling in mouse liver and human hepatocytes.* Manuscript.

IV. **Nerstedt A,** Cansby E, Andersson CX, Laakso M, Stančáková A, Blüher M, Smith U, Mahlapuu M. *Serine/threonine protein kinase 25 (STK25): a novel negative regulator of lipid and glucose metabolism in rodent and human skeletal muscle.* Diabetologia. 2012 Jun;55(6):1797-807.

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AMPK	AMP-activated protein kinase
AS160	PKB substrate of 160 kDa
ASK1	Apoptosis signal-regulating kinase 1
ATCC	American type culture collection
BHLHB	Basic helix-loop-helix domain containing class-B
BMAL1	Brain muscle arnt-like 1
CCM	Cerebral cavernous malformation
CKI	Casein kinase I
CLOCK	Circadian locomotor output control kaput
CRY	Cryptochrome
2-DG	2-Deoxyglucose
ER	Endoplasmatic reticulum
ERK	Extracellular-receptor kinase
FFA	Free fatty acid
GCK	Germinal center kinases
GLUT	Glucose transporter
HFD	High fat diet
HK2	Hexokinase 2
HP	Haptoglobin
IGT	Impaired glucose tolerance
IKK	IkappaB kinase
IL	Interleukin
IL6Rα	IL-6 receptor subunit α
INSR	Insulin receptor
IP	Intraperitoneally
IRS	Insulin receptor substrate
IR	Insulin resistance
JAK	Janus kinase
JNK	C-jun N-terminal kinase
LKB1	Liver kinase B1
LMO4	LIM domain only 4
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblasts
MM	Mismatch
MST	Mammalian sterile 20-like
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor-kappaB
NR1D	Nuclear receptor subfamily 1, group D
PAK	P21-activated kinase
PDK1	Phosphoinositide dependent protein kinase 1
PER	Period

PGC1-α	Peroxisome proliferator-activated receptor-gamma, coactivator 1-alpha
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinasephosphoinositide 3-kinase
PKB	Protein kinase B
PKR	Protein kinase R
PM	Perfect match
PPAR	Proliferator-activated receptor
RER	Respiratory exchange ratio
RISC	RNA-induced silencing complex
RORα	RAR-related orphan receptor-α
ROS	Reactive oxygen species
SAA	Serum amyloid A
SCN	Suprachiasmatic nuclei
SHP2	Src homology 2 domain containing protein tyrosine phosphatase 2
siRNA	Small interfering RNA
SIRT1	Sirtuin 1
SOCS3	Suppressor of cytokine signalling 3
SREBP-1c	Sterol regulatory element binding protein 1
STAT	Signal transducer and activator of transcription
STK25	Serine/threonine protein kinase 25
T2D	Type 2 diabetes
TNF-α	Tumor necrosis factor-α
UCP	Uncoupling protein
qRT-PCR	Quantitative real-time PCR

TABLE OF CONTENTS

INTRODUCTION

Type 2 diabetes and insulin resistance

Type 2 diabetes (T2D) is a complex metabolic disorder characterised by hyperinsulinaemia, hyperglycaemia and dyslipidaemia. The prevalence for T2D is growing and is expected to continue to grow due to population growth, aging, and increasing prevalence for obesity and physical inactivity. The total number of people with diabetes is expected to rise from 285 million in 2010 to 439 million in 2030 (1). Importantly, from being a disease of middle-aged and older subjects T2D has today emerged as a new and serious health problem in children (2; 3). Thus, with its steep increase in incidence, T2D is becoming one of the major global threats to human health. Globally, the total burden of diabetes and related complications is approximately 12% of the total health care costs (4). Epidemiologic studies and clinical trials have shown that T2D is largely preventable through diet and lifestyle modifications. However, to translate these findings into practice, fundamental changes in public policies and health systems are required (5).

More than 85% of people with T2D are overweight or obese and obesity is the major risk factor for development of insulin resistance (IR), which is a main predictor of T2D. The pathophysiology of IR is a reduced insulin sensitivity, which is an inability of insulin secreted by the β-cells in the pancreatic islets of Langerhans, to lower plasma glucose levels through suppression of hepatic glucose production and stimulation of glucose utilization in skeletal muscle and adipose tissue (6). As a result, the islet β-cells will increase the insulin secretion to maintain normal blood glucose levels. Ultimately, this will lead to β-cell dysfunction, resulting in insufficient levels of insulin secretion from these cells and manifestation of hyperglycaemia (7). Defects in the intracellular insulin signalling pathway emerge as having a central role for the origin of IR.

Intracellular insulin signalling and insulin resistance

Insulin secreted by the pancreas binds to the α subunit of the insulin receptor (INSR), a tyrosine kinase receptor, on the target tissue. This leads to autophosphorylation of tyrosine residues in the β subunit of the receptor, which is a key step for further signal transduction (8). Upon receptor activation several cellular targets such as the insulin receptor substrate (IRS) family of proteins, become tyrosine phosphorylated, with IRS1 and IRS2 being the most important mediators for carbohydrate metabolism (9). Next, phosphorylated tyrosines on IRS proteins serve as docking and activating site for phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), resulting in increased intracellular concentration of the phospholipid phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). This, in turn, activates phosphoinositide dependent protein kinase 1 (PDK1), which subsequently phosphorylates protein kinase B (PKB, also referred to

as AKT). PKB activation then triggers downstream effects of insulin in peripheral tissues such as increased glucose uptake in skeletal muscle through translocation of glucose transporter 4 (GLUT4, also referred to as SLC2A4) to the cell membrane and conversion of glucose to glycogen, inhibition of gluconeogenesis and glycogenolysis as well as increased glycogen synthesis in liver and increased lipogenesis and decreased lipolysis in adipose tissue (reviewed by (10), Fig. 1).

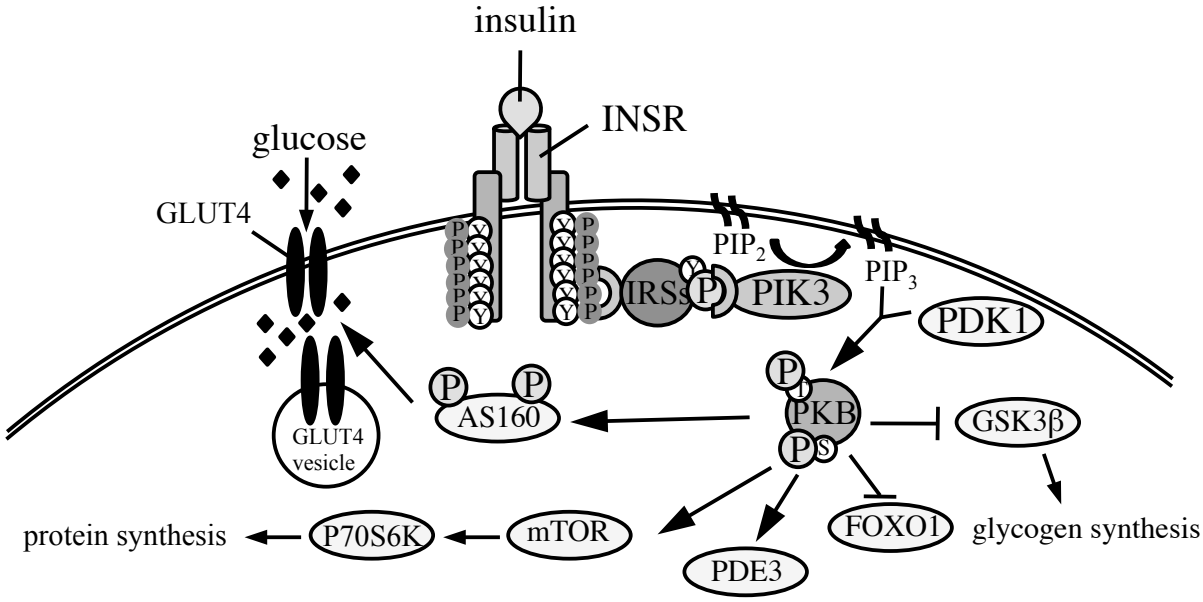

Figure 1. Overview of the insulin-signalling pathway. Binding of insulin to its receptor, results in tyrosine phosphorylation of IRSs, which will allow IRSs to associate with PI3K. PI3K activates PDK1, which in turn activates PKB. PKB phosphorylates numerous substrates, which are important for regulation of insulin dependent processes. PKB phosphorylates the PKB substrate of 160 kDa (AS160), which results in translocation of GLUT4 vesicles to the plasma membrane, facilitating the uptake of glucose into the cell. In addition, PKB deactivates glycogen synthase kinase 3 beta (GSK3β) leading to increased glycogen synthesis. PKB could also activate the mammalian target of rapamycin (mTOR)/p70 ribosomal S6 kinase (P70S6K)-signalling pathway, which will result in increased protein synthesis. Insulin-induced activation of phosphodiesterase 3 (PDE3) via PKB is a component of the antilipolytic action of insulin (11). Furthermore, PKB phosphorylates forkhead box O1 (FOXO1), which will result in its cytoplasmic retention and the repression of target gene expression, such as gluconeogenic genes (12). Adapted from (13).

It has become evident that IR is affecting insulin signalling at cellular level via postreceptor defects. There are numerous studies, both in patients as well as in experimental animals with IR, showing that protein level and activity of INSR is either normal or only slightly reduced and that this minor reduction is not sufficient to account for the substantially reduced insulin action observed. Several studies in IR animal models and humans have shown that an insulin signalling defect in the IRS-

PI3K-pathway accounts for IR phenotype in insulin target tissues (reviewed by (14)). In line with these studies, observations performed in humans show that rare mutations of IRS1 are associated with IR (15) and a dominant negative PKB2 mutation in man causes severe hyperinsulinaemia and diabetes (16). In addition to tyrosine phosphorylation, IRS proteins can be phosphorylated on serine residues, which attenuates insulin signalling by inhibiting insulin-stimulated tyrosine phosphorylation as well as by reducing the ability of IRS proteins to attract PI3K (reviewed by (14)). Serine phosphorylation of IRSs can also lead to increased degradation of the protein, thereby attenuating the insulin signalling prematurely. Normally, the regulatory subunit p85 of PI3K, which exists bound to the catalytic subunit p110 or as a free monomer, is in excess of p110. However, a disruption in the balance between the amounts of the two PI3K–subunits has been shown to potentially lead to IR in both animal models and in human studies (reviewed by (17)). Interestingly, elevated plasma levels of free fatty acids (FFA) and inflammatory cytokines can contribute to IR both by inducing serine phosphorylation of IRS1 and by activating phosphateses that negatively regulate the insulin-signalling cascade. FFAs increase the production of ceramides, lipids that act as second messengers, which activate protein phosphatase 2A (PP2A), leading to dephosphorylation and inactivation PKB (18). IR has lately been shown to associate with a state of low-grade chronic inflammation in metabolic tissues, and inflammation is strongly implicated in the development of IR.

Metabolically triggered inflammation

Recent evidence indicates that nutrient excess and obesity lead to chronic low-grade inflammation, also called metainflammation, in metabolic tissues. Metainflammation and inflammation, i.e. the classic way to look at inflammation, are distinguishable. The classical inflammation is described as a defence evoked by the body to injuries or infections. The hallmark of this response includes swelling, redness, pain and fever. This adaptive response is rapid and the insult is normally removed or neutralized fast and the inflammation is resolved. Even if metainflammation triggers a subset of signalling pathways and molecules involved in classical inflammation, it is a long-lasting condition primarily with harmful consequence for the metabolic milieu. Not only immune cells are engaged in metainflammation, but specialized metabolic cells such as adipocytes and hepatocytes.

Metainflammation is initiated predominately in adipose tissue, and is caused by excess consumption of nutrients. Nutrient overload triggers inflammatory-signalling pathways in adipocytes by activating intracellular kinases such as c-jun N-terminal kinase (JNK), IkappaB kinase (IKK), or protein kinase R (PKR). This will induce a moderate inflammatory response with a low-level induction of inflammatory cytokines such as tumor necrosis factor (TNF)-α, CC-chemokine ligand 2 (CCL2), interleukin (IL)-1β,

and IL-6. Increased cytokine secretion will in turn recruit inflammatory cells, mainly macrophages and T-lymphocytes, to the place of action, which will even further enhance the inflammatory cascade. All these events will be as a circuit without an end and thereby the inflammatory state will continue without an apparent resolution (reviewed by (19)). Different mechanisms have been suggested for onset of the inflammatory response evoked by nutrient overload. The demand on the endoplasmatic reticulum (ER) due to obesity as well as the production of reactive oxygen species (ROS) due to hyperglycemia will increase, which will trigger the activation of inflammatory pathways (reviewed by (20)).

The effects of metainflammation, once initiated within adipocytes, will proceed in liver, skeletal muscle, brain and pancreas, ultimately leading to metabolic dysfunction and IR at whole body level. Compared to adipose tissue, liver will not experience an infiltration of macrophages during the onset of obesity. Instead, secretion of inflammatory markers will take place within cells of the liver such as hepatocytes and macrophage-like Kupffer cells (21). In contrast, muscle cells are not known to secrete inflammatory signals in obesity. Instead inflammatory mediators secreted from liver and adipose tissue will affect skeletal muscle metabolism (Reviewed by (19)).

The mechanisms of how metainflammation interferes with intracellular insulin signalling contributing to IR remain largely unknown. It has been demonstrated that serine/threonine kinases, such as JNK, IKK, PKR and Protein Kinase C theta (PKCθ) triggered in response to inflammatory signalling contribute to inhibition of insulin signalling via serine phosphorylation of IRS1 (reviewed by (22), (23; 24)). Furthermore JNK, IKK and PKR can also induce the transcription of inflammatory mediators through activation of transcription factors, activator protein-1 (AP-1), nuclear factor-kappaB (NF-κB) and interferon regulatory factor (IRF), respectively (22; 23), which further promotes the inflammatory cascade. Furthermore, it is well known that the cytokine IL-6 secreted by liver and adipose tissue induces the janus kinases (JAKs), which leads to activation of the transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT3 (reviewed by (25)). STAT3 is not only inducing the expression of acute-phase response genes, such as serum amyloid A 1 (*SAA1*), *SAA2* and *haptoglobin* (*HP*) (26) but also the suppressor of cytokine signalling 3 (*SOCS3*) in liver (25; 27). Importantly, SOCS3 has recently been shown to repress the insulin signalling pathway by proteasomal degradation of IRS1 and IRS2, by binding to tyrosine residues on INSR, thereby inhibiting tyrosine phosphorylation of IRSs, and by inhibition of INSR tyrosine kinase activity (reviewed by (27)).

Circadian clock and metabolic regulation

The circadian rhythm is a 24-hour cycle in biochemical, physiological and behavioural processes. There is an essential connection between circadian regulation and metabolic function as perturbations of circadian clock are associated with an increased risk for the development of metabolic disorders in humans (28). Similarly, glucose intolerance and obesity develop in mice with defective circadian clock function (29; 30). Recent evidence indicates that disturbances in circadian rhythm are sufficient to induce the expression of proinflammatory cytokines such as TNF-α and IL-6, ultimately causing metainflammation (31).

The central circadian clock is located in the suprachiasmatic nuclei (SCN) of the hypothalamus (32). This master clock controls the daily sleep-wake cycle, body temperature, and feeding and activity behaviours. Circadian clocks in peripheral tissues are suggested to optimize the timing of metabolic processes to be able to efficiently store or utilize metabolites and many metabolic activities have been shown to act in a circadian manner with diurnal changes. Hormones, such as insulin, glucagon, adiponectin, leptin, and ghrelin show oscillating levels. Lipid and cholesterol biosynthesis, carbohydrate metabolism and transport, oxidative phosphorylation and detoxification pathways are regulated in a circadian manner by rate limiting enzymes. In fact, the fraction of cyclically expressed transcripts in peripheral tissues is as high as 5 to 20 % (33). It has been shown that circadian genes are expressed in a tissue specific pattern with <10% of the circadian genes in any tissue commonly expressed in another tissue (reviewed by (34)).

The master circadian pacemaker in hypothalamus is primarily entrained by light, which illuminates through a retinohypothalamic tract linking the retina to the SCN. The master clock can synchronize peripheral circadian clocks via chemical cues, such as rhythmically secreted hormones, or these peripheral circadian clocks could directly respond to the environment. Exercise can directly affect expression levels of key clock components and downstream targets in skeletal muscle (35). In animal studies, it has been shown that temperature cycles or feeding rhythm that oppose the natural rhythm can shift the phases of circadian oscillators in peripheral tissues (36-38). While the effects of the circadian clock on metabolic processes have now been well documented, much less is known about how metabolic processes may alter the circadian clock. The clock function in human neuroblastoma cells was found to be regulated by the redox state of nicotinamide adenine dinucleotide (NAD) (39), which is directly influenced by energy metabolism. Peroxisome proliferator-activated receptor-gamma, coactivator 1, alpha (PPARGC1A also referred to as PGC-1α), a transcriptional coactivator that regulates energy metabolism, influences the circadian clock in skeletal muscle and liver (40). Lately, new evidence suggests that metabolic energy sensors, such as AMP-

activated protein kinase (AMPK) (41-45) and sirtuin (SIRT1) (46-49), transmit energy dependent signals to the circadian clock.

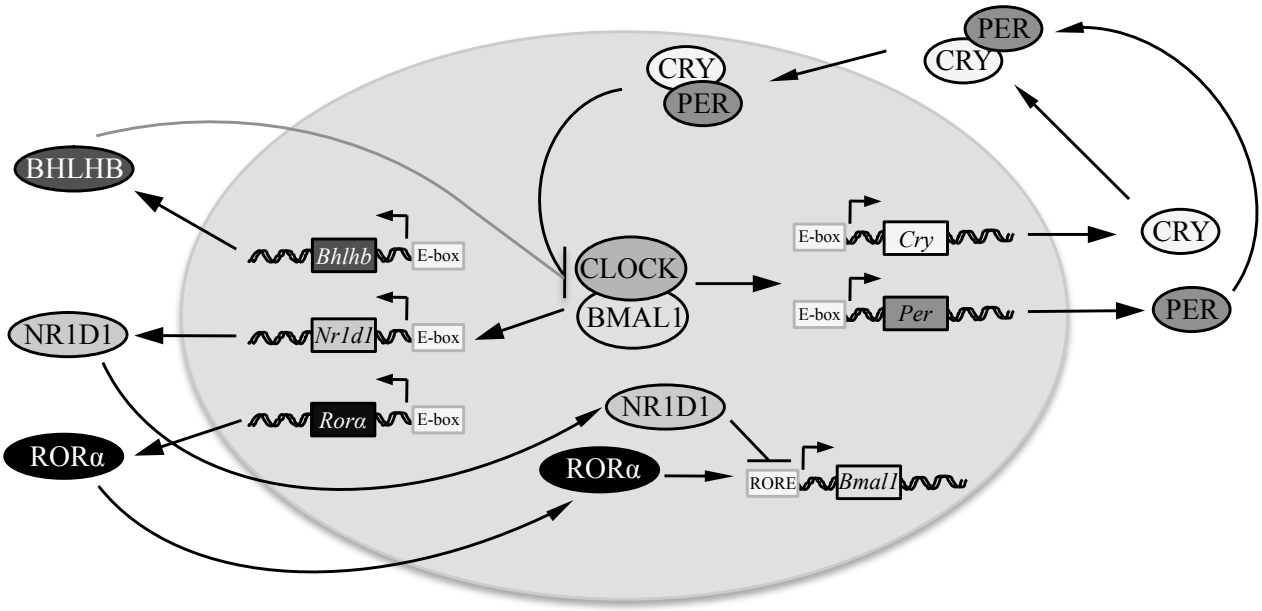

Figure 2. The core mechanism of the circadian clock. Adapted from (50). ROR elements, RORE.

The central and peripheral oscillators share a common molecular circuitry, with a battery of transcriptional activators and repressors forming a self-sustained transcriptional feedback loop (51; 52). The core of this mechanism is the circadian locomotor output control kaput (CLOCK) and brain muscle arnt-like 1 (BMAL1) proteins, which form a heterodimeric transcription factor complex (53). This complex will activate the transcription of target genes containing E-box regulatory enhancer sequences, including the period (*Per1, Per2,* and *Per3*) and cryptochrome (*Cry1* and *Cry2*) genes as well as the nuclear receptor subfamily 1, group D, member 1 and 2 (*Nr1d1* and *2,* also referred to as orphan nuclear receptors α and β *(Rev-Erb α* and *β*)) and the RAR-related orphan receptor-α (*Ror*α) genes. Following translation, PER and CRY proteins will dimerize and translocate to the nucleus where they will inhibit the CLOCK/BMAL1 complex and thereby repress their own transcription (reviewed by (54)). To further enhance the stability and precision of the circadian rhythm, additional loops are connected to the core clock, with NR1D1 repressing the transcription of *Bmal1*, RORα activating *Bmal1* expression, and the basic helix-loop-helix domain containing class-B 2 (*Bhlhb*2, also referred to as *Dec1*) and *Bhlhb*3 (also referred to as (*Dec2*)) repressing Clock and BMAL1 induced transactivation of PER ((55-57), Fig. 2). Only a few key metabolic genes are direct targets of the core clock genes (58; 59).

Instead, the majority of Clock targets genes are transcription factors, which will have an impact on the periodicity of downstream metabolic genes (51).

AMP-activated protein kinase (AMPK) and Serine/threonine protein kinase 25 (STK25) – two kinases regulating the metabolic homeostasis in liver and skeletal muscle

The focus of this thesis work is to describe novel molecular mediators of metabolic regulation, and possible connection to metainflammation and molecular clock function, in peripheral tissues, with the focus on two serine/threonine kinases – AMPK and STK25.

AMPK - evolution

AMPK was first described by Carling and Hardie in 1987 (60), and was thereafter substantially ignored for the next 10 years. After the first publications reporting the involvement of AMPK in glucose homeostasis in 1998 (61; 62), the general interest in this kinase has drastically grown, and in 2001 almost 250 papers were published with AMPK in the title or abstract. Furthermore, 10 years later the total number of published paper in the field of AMPK was raised to nearly 4000 (63).

AMPK is an evolutionarily conserved serine/threonine protein kinase and has been established to function as a major cellular energy sensor in all eukaryotic cells. This protein is activated by an increase in the AMP to ATP ratio within the cell and once activated it phosphorylates downstream substrates, which in general switches on catabolic pathways generating ATP while switching off ATP-consuming processes (64). In addition to the acute effect on cellular energy metabolism, AMPK also shows long-term effects on gene expression as well as protein production and modification. AMPK acts not only as a metabolic master switch by regulating fuel homeostasis at the cellular level but this protein also controls the whole body glucose homeostasis and insulin sensitivity.

Structure of AMPK complex

AMPK is a heterotrimeric enzyme composed of a catalytic α subunit and two regulatory subunits, β and γ, which all are required for its activity (65). The mammalian genome contains seven AMPK genes encoding for two α, two β and three γ isoforms and in total 12 heterotrimeric complexes could be formed. Upon activation, AMPK becomes phosphorylated on Thr^{172} on the α subunit by upstream kinases as the liver kinase B1 (LKB1, (66)), the calmodulin-dependent protein kinase kinase β (CAMKKβ, (67)), and the transforming growth factor-beta-activated kinase 1 (TAK1, (68)), which will cause >200-fold activation. Additionally, AMP can activate AMPK up to 10-fold by binding allosterically to the cystathionine-β-synthase (CBS) domains on the γ subunit (reviewed by (69), (70)). Dephosphorylation of AMPK on Thr^{172} of

the α subunit, which leads to inactivation of the kinase, is achieved by members of the phosphoprotein phosphatase family and metal-dependent protein phosphatase family (70; 71). It has also been suggested that AMP could increase Thr^{172} phosphorylation by protecting AMPK from dephosphorylation ((70; 72; 73), Fig. 3). More recently it has become evident that even ADP, can protect AMPK against dephosphorylation of Thr^{172} (74).

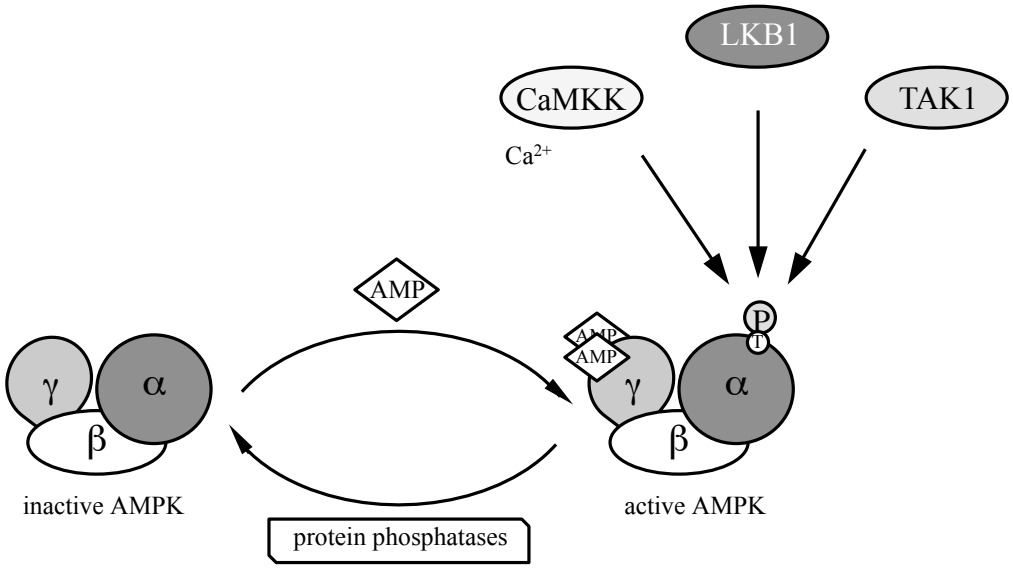

Figure 3. Regulation of AMPK by phosphorylation of Thr^{172} and by allosteric binding of AMP.

Various types of metabolic stresses such as glucose deprivation, hypoxia, ischemia, oxidative stresses and muscle contraction activate AMPK. These activation mechanisms, which involve increased cellular AMP, ADP or Ca^{2+} levels, are regarded as classical or canonical. However, recent studies indicate that AMPK could be activated by non-canonical pathways, which induce cellular stress triggered by ROS and DNA-damaging agents (reviewed by (75)).

AMPK in regulation of skeletal muscle metabolism

After food intake, when circulating insulin levels are high, skeletal muscle is the main site for glucose disposal. Activation of AMPK increases the glucose uptake in skeletal muscle either acutely by increased translocation of GLUT4, the main glucose transporter in skeletal muscle, to the plasma membrane by AMPK phosphorylating the PKB substrate of 160 kDa (AS160), or chronically by increasing the expression of GLUT4 (76; 77). In addition, increased glucose transport via GLUT1 (also referred to as SLC2A1) has been shown in response to AMPK activation (78). AMPK also acutely increases fatty acid oxidation by phosphorylating and thus inhibiting the activity of acetyl-CoA carboxylase 2 (ACC2), which will decrease the level of malonyl-CoA. Since malonyl-CoA is a potent inhibitor of fatty acids transport into the

mitochondria, this will ultimately lead to an increased β-oxidation rate (61; 79). In addition, AMPK promotes mitochondrial biogenesis and the expression of nuclear encoded mitochondrial genes by up-regulating the master regulator *Pgc1-α* ((80), Fig. 4).

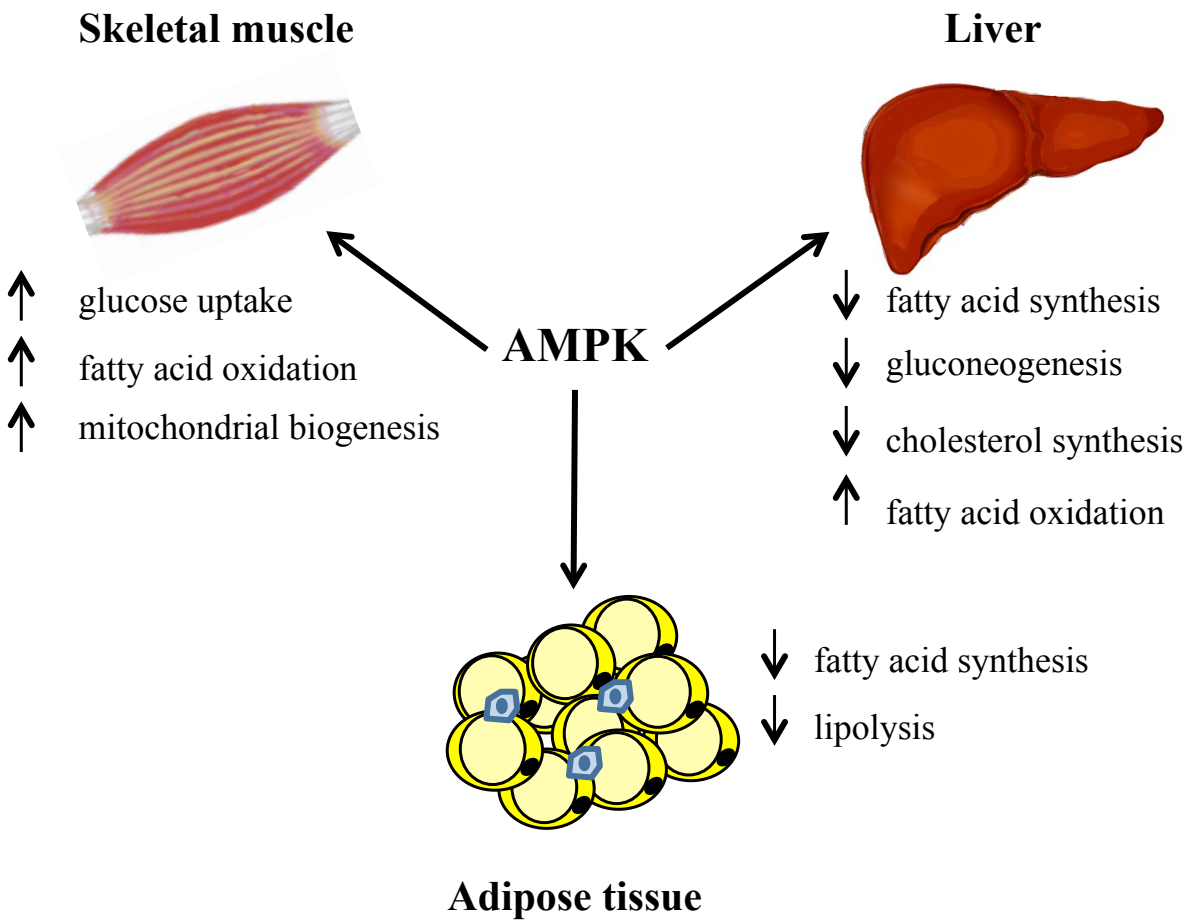

Figure 4. The effects of AMPK activation on skeletal muscle, liver and adipose tissue. Adapted from (81).

AMPK in regulation of liver metabolism

AMPK is regulating lipid oxidation, lipogenesis, and cholesterol synthesis in the liver. 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) and ACC1, key enzymes in cholesterol and fatty acid synthesis, respectively, are phosphorylated and inactivated by AMPK (64; 82). Also the transcription factor sterol regulatory element binding protein 1 (SREBP-1c) is phosphorylated by AMPK, leading to decreased expression of key enzymes in the fatty acid synthesis pathway (83). In addition, AMPK reduces the expression level of SREBP-1c as well as the carbohydrate response element-binding protein (ChREBP), transcription factors mediating activation of lipogenic and glycolytic genes, respectively (reviewed by (84)). AMPK is an important factor in controlling hepatic glucose production and has been shown to repress the expression of gluconeogenic genes such as glucose-6-

phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) by phosphorylation of the transcriptional coactivator, transducer of regulated CREB activity 2 (TORC2), which will not allow the coactivator to enter the nucleus (85). Thus, AMPK suppresses gluconeogenesis and lipid synthesis in the liver and also decreases lipid accumulation via increased lipid oxidation (Fig. 4).

AMPK in regulation of adipose tissue metabolism

In adipose tissue, activation of AMPK leads to decreased lipogenic rate, through inactivation of ACC by phosphorylation, as well as a decreased triglyceride synthesis depending on decreased glycerol-acyl transferase activity (reviewed by (86)). Furthermore, the hormone–sensitive lipase (HSL), a rate-limiting enzyme controlling lipolysis, is phosphorylated and thus inhibited by AMPK leading to decreased lipolysis (87). AMPK has also an inhibitory effect on the expression and secretion of two proinflammatory cytokines, TNFα and IL-6, in human adipose tissue. All these actions of AMPK will lead to decreased levels of circulating lipids and a reduced inflammatory response (86). In addition, chronic activation of AMPK in adipocytes increases the expression of PGC-1α, peroxisome proliferator-activated receptor (PPAR)α and PPARγ, transcription factors involved in mitochondrial biogenesis and oxidative metabolism ((88), Fig. 4).

Pharmacological activation of AMPK

Pharmacological activation of AMPK could be achieved by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), which is a cell permeable compound able to activate AMPK in various tissues. Once taken up by the cells, AICAR is converted to ZMP, an AMP analog, which mimics the effect of AMP by binding to the γ subunit and activating AMPK allosterically (89). There are several agents that are widely used in the treatment of T2D and that act by activating AMPK. Metformin (1,1-dimethylbiguanide), which has been used to treat diabetes for 50 years and is still the front-line drug, activates AMPK indirectly by inhibiting complex I of the respiratory chain, which will result in a lower ATP:AMP ratio (90). Secondly, thiazolidinediones (TZDs), including troglitazone, rosiglitazone and pioglitazone, which were introduced in T2D therapy in the late 1990s, activate AMPK by inhibition of complex I of the respiratory chain, but also by inducing the expression of adiponectin in adipocytes (91-93). Adiponectin, in turn, activates AMPK in liver and skeletal muscle (94). Even direct activators of AMPK have been developed as the compound A-76966, which binds to the β-subunit and activates AMPK both allosterically and by inhibiting dephosphorylation of Thr^{172} of the α subunit (95; 96). All of these compounds have shown to have off target effects and, therefore, it is of outermost importance to develop drugs with higher specificity towards the target molecule (reviewed by (97)).

AMPK and inflammation

Recent evidence points towards AMPK being an important regulator of inflammatory processes. The AMPK activator, AICAR, has been shown to inhibit inflammatory response in experimental models e.g. autoimmune encephalomyelitis (98), relapsing colitis (99), cystic fibrosis (100), asthma (101) and lipopolysaccharide (LPS)-induced lung injury (102). It has also been described that the AMPK agonist metformin can reduce circulating C-reactive protein (CRP) levels in T2D individuals. In addition, Sag *et al*. have shown that macrophages stimulated with anti-inflammatory cytokines (e.g. IL-10 and transforming growth factor β (TGF-β)) exhibit AMPK activation whereas cells treated with proinflammatory stimulus (e.g. LPS) show inactivation of AMPK, and that AMPK promotes macrophage polarisation towards an anti-inflammatory phenotype (103). Furthermore, *in vitro* studies performed in endothelial cells and macrophages have described that AMPK activation negatively regulates the inflammatory response evoked by different stimuli such as free fatty acids, LPS and TNF-α, by inhibiting NF-κB signalling (104-108). Reduced NF-κB activity as a result of pharmacological activation of APMK has also been shown in human muscle precursor cells from obese individuals (109). Recently, it was shown that the AMPK activator, metformin, could reduce IL-6-induced inflammatory response by decreasing the expression of the inflammatory marker *Saa1* in mouse liver (110).

STK25

Several molecular mediators for AMPK signalling have been identified in metabolic tissues. Our previous studies have shown that one differentially regulated gene in skeletal muscle comparing AMPKγ3 knockout mice to wild-type littermates is *Stk25*, which raised the hypothesis that this kinase might be involved in metabolic and inflammatory regulation by AMPK.

STK25 (also referred to as SOK1 and YSK1) belongs to the STE20 protein kinase superfamily, which includes more than 30 members. The *Ste20* (from "sterile) gene was first identified in the budding yeast *Saccharomyces cerevisiae* as a mitogen-activated protein kinase kinase kinase kinase (MAP4K) involved in the mating pathway. This superfamily is divided into two groups, the p21-activated kinases (PAKs) and the germinal center kinases (GCKs). These two groups are subdivided further into 10 subfamilies, PAK-I and -II and GCK-I to –VIII. The PAK family of kinases has a C-terminal kinase domain and an N-terminal binding site for small GTP-binding proteins while the GCK family has a N-terminal catalytic domain and a C-terminal regulatory domain. The STE20 superfamily of kinases is involved a wide range of biological responses such as regulation of cell proliferation, cell death, cytoskeleton rearrangements, and cell migration in response to extracellular stimuli or different forms of cellular stress. Most of the members of this family have been shown

to be capable of activating MAPK pathways such as the extracellular signal-regulated protein kinase (ERK) pathway, the JNK pathway and the p38 MAPK pathway. According to the phylogenetic tree published by Dan *et al*. 2001, STK25 belongs to the GCK-III sub-family of proteins together with mammalian sterile 20-like 3 (MST3) and MST4 (also referred to as MASK,(111)).

The serine/threonine protein kinase STK25 was first cloned and characterized in 1996 by Pombo *et al*. (112) and it was shown to be broadly expressed in rodent tissues such as brain, heart, intestine, kidney, liver, lung spleen and skeletal muscle (113). STK25 is a protein comprising 426 amino acids with a calculated molecular weight of 48 kDa. However, the apparent molecular mass on SDS-polyacrylamide gel electrophoresis is 55 kDA, which probably reflects on post-translational modifications of the protein (113). STK25 is regulated by phoshporylation/dephosphorylation events, with autophosphorylation of Thr^{174} being an important mechanism for activation of the kinase activity. The C-terminal regulatory domain of STK25 has an inhibitory effect on the kinase activity (112). STK25 is activated by oxidative stress and chemical anoxia, with reactive ROS being the primary mechanism, but not by any other environmental stresses or growth factors (112; 114). Interestingly, STK25 is not activating any of the known MAPK pathways including ERK, JNK and p38, as described for other family members (112; 113).

STK25 has been shown to associate with the Golgi matrix protein, GM130, which will render STK25 active by autophosphorylation and once activated STK25 phosphorylates the 14-3-3ζ-protein to implement normal cell migration and polarization (115). The interaction between STK25 and GM130 is stabilized via the cerebral cavernous malformation 3 (CCM3, also referred to as PDCD10), a protein known to be important for normal cardiovascular development *in vivo*. This interaction protects STK25 from ubiquitin ligation as well as preserves the kinase activity of STK25 (116; 117). In addition, STK25 acts as a scaffolding protein by linking LKB1-STE20-related adaptor (STRAD) to GM130 to regulate Golgi morphology and neuronal polarization (118). In response to chemical anoxia and the following ROS production, STK25 is cleaved by caspases and thereby it dissociates from the Golgi complex and translocates to the nucleus inducing apoptotic cell death (119). However, STK25 could also induce cell death following oxidant stress by exit the Golgi complex, disrupting the 14-3-3ζ/apoptosis signal-regulating kinase 1 (ASK1) complex by phosphorylation of the 14-3-3ζ-protein, which leads to ASK1-mediated cell death ((120), Fig. 5).

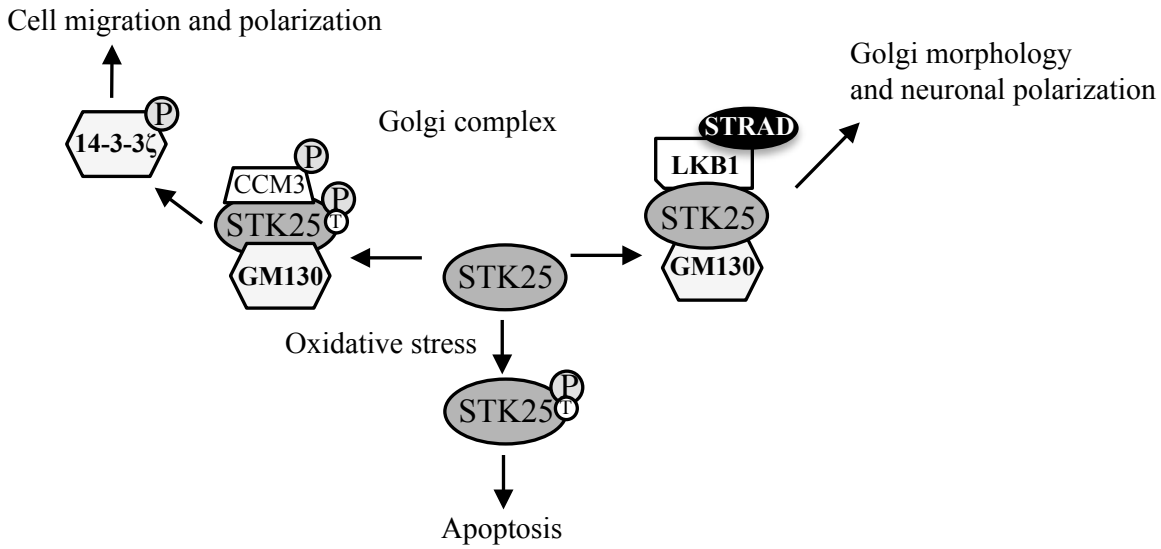

Figure 5. STK25 and cellular functions. Adapted from (121).

MST3 and MST4 are the nearest neighbours of STK25. MST3, as STK25, can be cleaved by caspases to promote apoptosis and MST3 could also inhibit cell migration by inactivating the protein tyrosine phosphatase – proline-, glutamic acid-, serine- and threonine-rich (PTP-PEST), a scaffold protein tyrosine phosphatase. Furthermore, it controls the cell shape and cell cycle by phosphorylating the nuclear Dbf2-related kinase 1 (NDR1), which is implicated to regulate cell proliferation, tubulin cytoskeleton organization and polarization. MST4 can activate ERK to induce cellular transformation, which could be further enhanced by MST4 interacting with CCM3. In addition, MST4 interacts with GM130, which will restrict MST4 at the Golgi matrix to control cell migration (reviewed by (121)).

AIM

The overall aim for this thesis was to characterize the signalling pathways for the metabolic master switch, AMPK, to control metabolic and inflammatory cross-talk and to elucidate the impact of a novel kinase STK25 in the regulation of skeletal muscle metabolism.

Specific aims:

Paper I: To study the impact of pharmacological activation of AMPK *in vivo* on circadian clock function, comparing AMPKγ3 knockout mice to wild-type littermates using a global gene expression analysis approach.

Paper II: To investigate the role of AMPK in the regulation of the inflammatory response evoked by cytokines in human hepatocytes.

Paper III: To investigate the mechanism for AMPK to interfere with STAT3 signalling in human hepatocytes and to evaluate the capacity of AMPK activation to repress liver inflammation *in vivo.*

Paper IV: To characterize the role of STK25 in the regulation of skeletal muscle metabolism.

EXPERIMENTAL PROCEDURES

Experiments involving human subjects

The gene expression of *STK25*, uncoupling protein 3 (*UCP3*), and hexokinase 2 (*HK2*) in *s*keletal muscle biopsies from 41 consecutively recruited Caucasian men (n=23) and women (n=18) were analysed. The subjects were categorized into groups of normal glucose tolerance (NGT) (n=13, 9 males, 4 females), impaired glucose tolerance (IGT) (n=14, 8 males, 6 females), and T2D (n=14, 6 males, 8 females) based on a 75 g oral glucose tolerance test. Patient recruitment and collecting biopsies were performed in collaboration with Prof. M. Blüher, Leipzig University, Germany. This study was approved by the ethics committee of the University of Leipzig and conformed to the Declaration of Helsinki and all participants provided written informed consent before taking part in the study. For more information regarding the outline of the human study, see Supplemental Materials for *Paper IV*.

Animal experiments

In *Paper I*, AMPKγ3 knockout (*Prkag3*$^{-/-}$) mice and wild-type littermates were used. *Prkag3*$^{-/-}$ mice were created by conventional gene targeting methods, which led to inactivation of AMPKγ3 in all tissues (122). Since the AMPKγ3 subunit is selectively expressed in skeletal muscle (123), the primary effect of the gene targeting was expected to be seen in this tissue. The mice were bred into C57BL/6 genetic background. The local ethical committee in Stockholm approved the experiments performed. In this study, AMPKγ3 knockout mice and wild-type littermates were injected intraperitoneally (IP) with either saline (0.9% NaCl) or AICAR (0.25 g/kg). Whole body energy homeostasis was evaluated in *Prkag3*$^{-/-}$ mice and wild-type littermates using metabolic cages. Mice were housed individually in metabolic cages and followed for 120 h with automated recordings every 20 min. Oxygen consumption, locomotor activity and respiratory exchange ratio (RER) were measured. Animal experiments in *Paper I* were performed in collaboration with Prof. J. Zierath, Karolinska Institutet, Stockholm, Sweden. For further information, see *Paper I.*

For the *in vivo* experiments in *Paper III,* C57BL/6 male mice at 8-9 weeks of age were used. Animal experiments were performed with the approval of the local Ethics Committee for Animal Studies at the Administrative Court of Appeals in Gothenburg, Sweden. In this study, C57BL/6 mice were injected IP with either saline (0.9% NaCl) or AICAR (0.25 g/kg), followed 1 h later by injections of saline or human rIL-6 (0.5 μg/animal). 15 or 45 min after the last injection, liver was dissected and both RNA and protein were extracted.

Cell culture experiments

Cell lines

The human cell line, HepG2 (hepatocellular carcinoma, human, American Type Culture Collection (ATCC), Manassas, VA, USA) has been derived from the liver tissue of a 15-years-old Caucasian American male with a well-differentiated hepatocellular carcinoma (124). The morphology of this immortalized cell line is epithelial and it has been widely used as a model system for studies of liver metabolism and drug targeting. It has also been shown that IL-6 could trigger an acute-phase response in HepG2 cells (125). HepG2 cells were cultured as described in *Papers II* and *III.*

The rodent cell line, L6 (skeletal muscle, *Rattus norvegicus*, ATCC) has been isolated from primary cultures of rat thigh muscle. Under normal growth conditions these cells propagate as mononucleated myoblasts. Once L6 cells become confluent, they start to differentiate into multinucleated myotubes (126). L6 cells were used for metabolic studies and they were cultured as described in *Paper IV*.

Primary hepatocytes isolated from mouse liver are effective tools to confirm results gained using immortalized cell lines. These cells are cultured on collagen-coated plates, and thus, they will preserve both liver-specific functions and morphology over a substantial period of time (127). Primary hepatocytes were cultured and treated as described in *Paper II*.

Transient transfection of cell lines

In *Paper IV,* L6 myoblasts were transiently transfected with a FLAG-tagged rat Stk25 construct using Lipofectamine 2000 (Invitrogen, San Diego, CA, USA), a liposome-containing reagent. A liposome is a vesicle composed of a lipid bilayer. These positively charged vesicles aggregate with the negatively charged plasmid DNA and easily fuse with the negatively charged plasma membrane of living cells, thus delivering the construct into the target cells.

In *Paper II*, HepG2 cells were transfected with small interfering RNA (siRNA) interfering with the $\alpha 1$ and $\alpha 2$ subunit of AMPK. These transfections were performed using the lipid-mediated reagent Lipofectamine RNAiMax (Invitrogen). siRNAs, once introduced into the cell, will associate with a protein complex called the RNA-induced silencing complex (RISC). The RISC complex containing the siRNA, which is complementary to the target mRNA, recognises and binds the target mRNA and mediates the degradation of mRNA (reviewed by (128)). In *Paper IV,* L6 myoblasts were transfected with siRNA interfering with STK25. For further information regarding siRNA concentrations and cell densities see *Papers II* and *IV*.

In vitro cytokine stimulation

HepG2 cells were stimulated with human recombinant cytokines such as IL-6, IL-1β, and TNF-α as described in *Papers II* and *III*. Briefly, 16 h before cytokine stimulation, ordinary growth medium was exchanged to starvation medium and the cells were treated with AMPK agonists, AICAR or metformin, at concentration and time points indicated in *Papers II* and *III*. Both RNA and proteins were extracted from these cells.
In *Paper II*, primary mouse hepatocytes were pre-treated with AICAR (2 mmol/l) or metformin (5 mmol/l) 16 h before mouse rIL-6 (20 ng/ml) was added for 20 min. Proteins were extracted from these cells.

Analysis of mRNA and protein expression

RNA extraction

In *Paper I*, the RNeasy Fibrous Mini Kit (Qiagen, Valencia, CA, USA) was used for RNA preparation from skeletal muscle. It is known that RNA purification from fibrous tissues, such as skeletal muscle, is difficult due to the abundance of collagen and contractile proteins. RNeasy Fibrous Mini Kit is provided with proteinase K, which digests these proteins. For tissue disruption the Mixer Mill MM 301 (Retsch, Haan, Germany) was used. In *Papers II* to *IV*, RNA was isolated with the RNeasy Mini Kit (Qiagen) (*Paper II*) or the EZNA Total RNA kit (Omega Bio-Tek, Norcross, GA, USA) (*Papers III and IV*).

Microarray analysis

For the global gene expression study described in *Paper I*, the GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA) were used, which will allow for analysis of over 39,000 transcripts on a single array. Each transcript is detected with a probe set, which is a collection of 16 to 20 pairs of probes (synthetic 25-mer oligonucleotides) synthesized on a coated quartz surface. Each pair of probes consists of two oligonucleotides – one perfect match (PM), complementary to the target mRNA sequences and one mismatch (MM), in which the middle nucleotide has been changed to its complement. The PM probe is gene specific and the MM probe is used to correct for non-specific binding of the mRNA. The sequences from which these probe sets are derived are selected from different databases such as GenBank®, dbEST, and RefSeq. For further information regarding the cRNA preparation and gene chip hybridization, see *Paper I*.
The gene array data were analyzed using GeneTrafficUNO version 3.2-11 (Iobion Informatics, La Jolla, CA, USA), and Spotfire Decision Site version 8.1 (TIBCO Software Inc., Palo Alto, USA). The GC-Robust Multi-array Average (RMA) algorithm was used to calculate the intensities for every probe set. The performance of the data analysis is outlined in *Paper I*.

Quantitative real-time PCR (qRT-PCR)

cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using ABI Prism 7900HT Sequencing Detection System (Applied Biosystems). Primers and probes were designed using the Primer Express software (Applied Biosystems). Both SYBR Green (*Paper I*) and TaqMan (*Papers II* to *IV*) chemistries were used.

For the SYBR Green based detection, SYBR Green I dye is used, which is a highly specific double stranded DNA-binding dye. The first step will be to denature the DNA to reduce the fluorescence. Then the temperature is reduced to allow for the primers to anneal to the template. Taq DNA polymerase is used for the extension of the target sequence, which will create the PCR product (amplicon). The SYBR Green dye will bind to each new copy of the amplicon and as the PCR reaction progresses more amplicons will be produced. This will be monitored as an increase in fluorescence intensity, which is proportional to the amount of PCR product produced.

TaqMan based detection uses a fluorogenic probe, which is specific for the gene of interest. The probe is conjugated with a reporter fluorescent dye at the 5'end and a quencher dye at the 3'end. The quencher dye will eliminate the fluorescence from the reporter dye as long as the probe is intact. Since the probe will anneal downstream of one of the primer binding sites, the 5'end nuclease activity of the DNA polymerase will cleave the probe when the primer extends. During the extension the reporter dye is cleaved from the probe and thus, it starts to emit its characteristic fluorescence. The signal from reporter dye is directly proportional to the amount of amplicon produced.

Quantification of the amount of target could be done with the standard curve method, using a serial dilution of a plasmid for the gene of interest, or with the comparative threshold method (2^{-Ct} method), using an endogenous housekeeping gene for normalization. The amount of specific transcript is measured at the exponential phase of the amplification before reaction components become limiting. Threshold cycle (Ct) is defined as the number of cycles for the fluorescent signal to be higher than the minimal detection level (129). This will always occur at the exponential phase and Ct values are inversely proportional to the amount of target transcript in the sample. The standard curve method was used in *Paper I* while the comparative threshold method was used in *Papers II* to *IV*.

Protein extraction and western blot analysis

Western blot analysis uses specific antibodies to identify proteins that have been separated from one another according to their size by gel electrophoresis.

In *Paper I*, the extensor digitorum longus (EDL) muscles were homogenized and protein lysates were prepared as described. To detect proteins of interest, western blot analysis was performed at the laboratory of Prof. J. Zierath, Karolinska Institutet,

Stockholm, Sweden for details see *Paper I*. In *Papers II* to *IV*, whole cell extract was prepared from HepG2 and L6 cells according to the description in *Paper II* and western blot was performed. Briefly, protein lysates were prepared and the protein concentration was determined in duplicates using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). To ensure that the transfer of proteins to the membrane was complete, the membrane was incubated with 0.5% Ponceau S solution, which is a rapid and reversible staining method for locating protein bands on western blots. Apart from that, housekeeping genes, such as actin or glyceraldehyde 3-phosphate dehydrogenas (GAPDH), were routinely used as loading controls. However, it has been shown that Ponceau S staining is equally good to actin as quality and loading control in western blotting (130). For detailed description of the western blot performance see *Papers II* and *IV*. For antibodies used, see respective *Papers*.

Glucose transport and palmitate oxidation assay

In L6 myoblasts, transfected with siRNA interfering with STK25, the glucose transport rate into the cells was measured using the glucose analogue 2-deoxyglucose (2-DG). 2-DG is phosphorylated by hexokinase to 2-DG-P but cannot be further metabolized. Thus, the rate of intracellular accumulation will reflect the transport and is not influenced by subsequent steps in the glucose metabolism (131). For further details, see Supplemental Materials for *Paper IV*.

Beta-oxidation is the process by which fatty acids are broken down in the mitochondria or peroxisomes to generate acetyl-CoA, which will be fed into the citric acid cycle to generate the high-energy molecule ATP, CO_2 and H_2O. Palmitate was used as substrate to measure the beta-oxidation rate in L6 myoblasts transfected with siRNA interfering with STK25. In this assay, ^{3}H-palmitate was used by the cells to generate ^{3}H-labeled H_2O as the final beta-oxidation product, which is released into the media and measured. For further details, see Supplemental Materials for *Paper IV*.

Statistics

Statistical analyses were performed using the Microsoft Excel. Statistical significance between the groups was calculated with an unpaired Student's *t* test, with a value of $p < 0.05$ considered statistically significant.

PAPER I

Summary of results

A global gene expression analysis was performed in white gastrocnemius muscle of wild-type and *Prkag3*$^{-/-}$ mice. In both genotypes, the impact of AMPK agonist AICAR administration was evaluated. One functional group of genes differentially regulated in AMPKγ3 knockout and wild-type mice were genes regulating the circadian clock. We found the expression level for *Cry2* to be significantly increased, and for *Bhlhb2* and *Nr1d1* to be significantly decreased in wild-type mice in response to AICAR, compared to saline ($p<0.01$, $p<0.01$, $p<0.05$, respectively). These genes were not affected by AICAR treatment in *Prkag3*$^{-/-}$ mice (Fig. 6). We also compared basal expression level of circadian core clock genes between *Prkag3*$^{-/-}$ mice and wild-type littermates. The only clock gene to be significantly changed was *Per1*, which was increased in knockout mice ($p<0.05$).

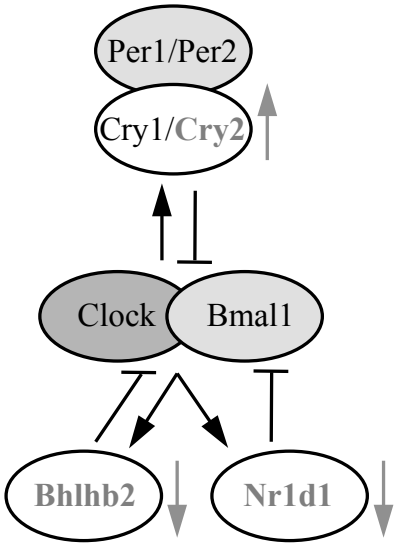

Figure 6. Cross-talk between genes involved in regulation of circadian rhythm. The positive loop of the circadian rhythm is formed by the CLOCK:BMAL1 heterodimer. This complex induces transcription of the negative arm of the core clock comprised of *Per* and *Cry* genes. PER and CRY proteins directly associate with CLOCK:BMAL1 and abrogate the transcriptional activity of the heterodimer. There are additional loops connected to the core clock, with NR1D1 repressing the transcription of *Bmal1* and BHLHB2 repressing CLOCK and BMAL1 induced transactivation of PER. Grey arrows indicate transcripts that are specifically up- or down-regulated, in wild-type but not in Prkag3$^{-/-}$ mice after AICAR treatment.

The effect of AICAR on the expression of core clock genes in wild-type mice suggests that AMPK influences the circadian function. To evaluate the role of AMPK on the physiological night/day rhythm, the respiratory exchange ratio (RER) was calculated

as the ratio between CO_2 production and O_2 consumption over a 24-hour period. Wild-type mice showed an increased RER during the dark period compared to the light period ($p<0.001$), which was expected and corresponds to a switch in fuel source from fat to carbohydrate. Interestingly, for *Prkag3*$^{-/-}$ mice no light-dark cycle variation of RER was observed, further indicating a possible role of AMPK in regulation of the circadian rhythm.

Discussion

It was shown by Um *et al*. that pharmacological activation of AMPK phosphorylates casein kinase I epsilon (CKIε), a key regulator of the circadian period length, in Rat-1 fibroblasts. Once activated CKIε phosphorylates PER, which leads to proteasome-mediated degradation of PER. Thus, AMPK activation leads to degradation of PER2 and a phase advance in the expression pattern of clock genes (41). Furthermore, Um *et al*. showed that metformin induced a phase advance in the expression of *Per1* and *Per2* in mouse skeletal muscle. This is in line with our investigation suggesting that AMPK regulates circadian rhythms in skeletal muscle. After these two studies were published, additional evidence has been provided supporting the role of AMPK in the regulation of circadian gene function and expression. Pharmacological activation of AMPK directly phosphorylates CRY1, thereby facilitating its degradation, in mouse embryonic fibroblasts (MEF) as well as in mouse liver (43). In line with this, AMPK activity was rhythmic and inversely correlated with the abundance of CRY1 (43). Lamia *et al*. followed the rhythmic behaviour of *Nr1d1* in synchronized MEFs after glucose deprivation or AICAR treatment, which both resulted in an increased expression of *Nr1d1* ultimately leading to a decreased *Bmal1* expression (43). However, Barnea *et al*. showed that treating lean, healthy mice with the AMPK agonist metformin affects the circadian clock in a tissue-specific manner with the activation of liver CKIα leading to a phase advance, while at the same time activating of muscle CKIε leads to a phase delay in the expression of clock genes (45). The rhythmic expression of the circadian core clock genes, *Clock, Bmal1, and Per2* in heart and skeletal muscle was severely affected in AMPKα2$^{-/-}$ mice and the same was found in white adipose tissue of AMPKα1$^{-/-}$ mice, further indicating that AMPK is involved in the circadian regulation in both tissue- and isoform-specific manner (41; 44). Notably, one additional mechanism for AMPK to interfere with the circadian clock is to enhance SIRT1 activity by increasing intracellular levels of NAD^+, leading to deacetylation of PER2, which ultimately will result in phosphorylation and degradation of PER2 (132; 133). In addition, impaired function of AMPK and SIRT1 together with decreased amount of CLOCK and BMAL1 seen in white adipose tissue from *db/db* mice, a genetic model for IR and obesity, was reversed after treatment with metformin (134). It has also been shown that mice fed a time-restricted high fat diet

(HFD) compared to mice with free access to HFD have improved AMPK function as well as improved oscillations of circadian clock components in the liver (135).

Conclusion

Taken together, our results indicate that pharmacological activation of AMPK regulates expression of several circadian clock genes in skeletal muscle of wild-type mice in an AMPKγ3 subunit-dependent manner, and that the diurnal shift in substrate utilisation seen in wild-type mice is blunted in *Prkag3*$^{-/-}$ mice. Thus, APMK, as a central energy sensor, could be one important node of linking energy metabolism to the circadian clock function.

PAPERS II and *III*

Summary of results

Liver is one of the metabolic tissues involved in the inflammatory response evoked by increased caloric intake. To elucidate the impact of AMPK on hepatic metainflammation, we performed both *in vivo* and *in vitro* experiments (*Papers II* and *III*). In *Paper II,* HepG2 cells were pre-treated with AICAR (2 mM) and metformin (5 mM), and then exposed to IL-6 (10 ng/ml). We showed that the IL-6-induced inflammatory response, estimated as the markedly increased expression of the acute-phase proteins *SAA1, SAA2* and *HP*, was significantly decreased with AICAR ($p<0.05$, $p<0.01$, $p<0.01$, respectively) and metformin ($p<0.01$) in dose-dependent manner in HepG2 cells. We also reproduced this in mice injected with AICAR before inflammation was induced by systemic IL-6 administration (*Paper III*). In this experiment, liver was dissected and the expression levels of *Saa1, Saa2* and *Hp* were analysed. AICAR showed an anti-inflammatory capacity *in vivo* by significantly inhibiting IL-6-induced expression of *Saa1* and *Saa2* ($p<0.01$).

AICAR and metformin are not specific activators of AMPK. Therefore, to elucidate the contribution of AMPK in the anti-inflammatory response evoked by AICAR and metformin, the catalytic $\alpha1$ and $\alpha2$ subunits of AMPK were repressed approximately 80% by siRNA transfection in HepG2 cells (*Paper II*). The inhibitory effect of AICAR and metformin on IL-6-induced expression of *SAA1* and *SAA2* was significantly lower in cells transfected with siRNA against the $\alpha1$ and $\alpha2$ subunits of AMPK (AICAR: $p<0.01$, $p<0.05$, respectively; metformin: $p<0.05$).

To map the mechanism for AMPK to interfere with IL-6 signalling, we characterized the effect of pharmacological activation of AMPK at total protein and phosphorylation level of all the components in the IL-6/STAT3 signalling pathway by pre-treating HepG2 cells with AICAR (2 mM) and metformin (10 mM), before IL-6 was added (*Papers III*). We demonstrate that both AICAR and metformin significantly decrease early IL-6-induced increase in $Tyr^{1022/1023}$ phosphorylation of JAK1 ($p<0.05$, $p<0.01$, respectively), Tyr^{542} phosphorylation of the SH2-domain containing protein tyrosine phosphatase 2 (SHP2) ($p<0.01$) and Tyr^{705} phosphorylation of STAT3 ($p<0.01$). The protein level of IL-6 receptor subunit α (IL6Rα) or Ser^{727} phosphorylation of STAT3 were not affected by AICAR or metformin. In addition, we show that AICAR, but not metformin decreases $Tyr^{1007/1008}$ phosphorylation of JAK2 ($p<0.01$), and that metformin, but not AICAR, reduces the protein level of GP130 approximately 3-fold ($p<0.05$). Furthermore, AICAR, but not metformin, reduces the protein level of LIM domain only 4 (LMO4), which has been suggested to stabilize the IL-6 receptor complex, by approximately 2-fold ($p<0.01$, Fig. 7).

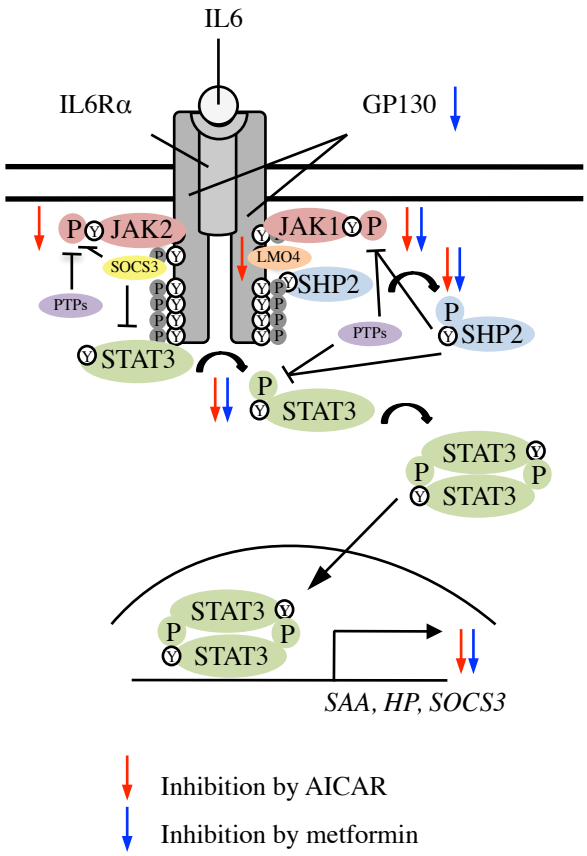

Figure 7. A schematic model of the effects of AICAR and metformin on the IL-6 signalling pathway. IL-6 signals through its receptor IL6Rα, which leads to the assembly of the IL6R/GP130 receptor complex. This receptor complex is further stabilised by LMO4. The formation of the receptor complex leads to phosphorylation and activation of receptor-associated JAKs, followed by phosphorylation of the signal transducing receptor subunit GP130. Once activated, GP130 will serve as docking sites for STAT3 and SHP2, which both will be phosphorylated by JAKs. Phosphorylated STAT3 will dimerize and translocate to the nucleus to activate transcription of target genes. JAKs as well as STAT3 proteins are negatively regulated by protein tyrosine phosphatases (PTPs). Red and blue arrows indicate targets, which are regulated at the level of phosphorylation or protein expression by AICAR and metformin, respectively. IL-6/STAT signalling pathway is summarised as described by Heinrich *et al*. (136).

To confirm that AMPK exerts its anti-inflammatory effect by similar mechanism *in vivo,* IL-6-induced phosphorylation of STAT3 was investigated in liver after IP injection of AICAR or placebo (*Paper III*). In line with *in vitro* data, AICAR reduced IL-6 induced Tyr^{705} phosphorylation of STAT3 in mouse liver ($p<0.05$). In addition, the expression level of *SOCS3*, a direct target of the transcription factor STAT3, was decreased in HepG2 cells and mouse liver after AICAR treatment ($p<0.01$).

Discussion

IR is a major metabolic feature of obesity and is also a main predictor of T2D. Recent evidence indicates that nutrient excess and obesity lead to chronic low-grade inflammation in metabolic tissues, which further promotes IR. Increased levels of pro-inflammatory cytokines, such as IL-6, TNF-α and IL-1β, show an association with obesity-induced IR with circulating IL-6 demonstrating the strongest relationship (137). IL-6 produced by adipose tissue has been shown to act in an endocrine manner in contrast to TNF-α, which may act locally in autocrine and paracrine manners (138). However, IL-6 is not only produced by adipocytes and various proinflammatory cells (reviewed by (139)), but also by many other cell types, such as hepatocytes and skeletal muscle cells (140; 141).

IL-6 had been shown to activate AMPK both in skeletal muscle and adipose tissue (reviewed by (142)). Interestingly, IL-6 knockout mice show a decreased AMPK activity in both muscle and adipose tissue while in liver the AMPK activity was equal to control mice (143). This is line with our results showing that IL-6 had no impact on AMPK activity in HepG2 cells (*Paper II*). Acute-phase proteins SAA1, SAA2 and HP, are synthesized in hepatocytes and upregulated in response to inflammatory signals evoked by cytokines such as IL-6 (reviewed by (144)). Consistently, our studies described in *Papers II* and *III* demonstrate a markedly enhanced expression of *SAA* cluster genes as well as *HP* in HepG2 cells and mouse liver in repose to IL-6 treatment. Interestingly, we found this acute phase gene expression evoked by IL-6 to be blunted by the AMPK agonists, AICAR and metformin, which had not been reported previously.

The cytokine IL-6 activates the JAK/STAT signalling pathway, which ultimately leads to tyrosine phosphorylation of STAT3 and the subsequent translocation of STAT3 into the nucleus inducing transcription of several target genes such as acute-phase response genes and SOCS3. This has been previously demonstrated in HepG2 cells (145) and is consistent with our results. (*Papers II and III*). In *Paper III* we show that IL-6-stimulated phosphorylation of key mediators of canonical IL-6 signalling - JAK1, and its targets, SHP2 and STAT3 is inhibited by both AICAR and metformin, suggesting that AMPK activation represses IL-6 signalling through inhibition of JAK1 phosphorylation. Thus, we have identified the point of interaction for AMPK activation with canonical IL-6 signalling pathway in liver.

It has been shown that the hepatic target of IL-6/STAT3 signalling, SOCS3 is a mediator of IR in liver. SOCS3 was shown to repress the insulin signalling pathway by inhibiting tyrosine phosphorylation of IRS1 and IRS2 by competitive binding at the docking site on INSR, by inducing serine phosphorylation of IRS1 and IRS2 and thereby marking them for proteasomal degradation, and by inhibition of INSR tyrosine kinase activity (reviewed by (27)). Exposure to palmitate, a saturated fatty acid, leads

to constitutively phosphorylated STAT3, and corresponds to increased amount of SOCS3 and reduced insulin-stimulated activation of PKB in L6 myotubes (146).
Recently, it was shown that metformin improves IL-6-induced IR by inhibition of the IL-6/STAT3 signalling pathway via increasing the orphan nuclear receptor small heterodimer partner (SHP, also referred to as NROB2) protein in rat primary hepatocytes, which will repress the transcriptional activity of STAT3 by direct interaction and repression of the DNA binding capacity of STAT3 on the *Socs3* gene promoter (110). Furthermore, pharmacological activation of PPARβ/δ attenuates IL-6-induced IR in hepatic cells by preventing STAT3 phosphorylation and the following increase in SOCS3 through inhibition of ERK1/2 phosphorylation and prevention of IL-6-induced reduction in AMPK activity (147). In other cells types, such as macrophages, endothelial cells, and neutrophils, AMPK has been shown to interfere with cytokine signalling by repressing mediators of the NF-κB pathway. We have not evaluated the possible involvement of NF-κB in AMPKs effect in liver cells, which will be the focus of our further studies.

Conclusion

Pharmacological activation of AMPK suppresses IL-6-induced acute-phase response in mouse liver and hepatocytes by inhibiting IL-6-stimulated phosphorylation of downstream mediators of the canonical IL-6/STAT3 signalling pathway – JAK1, SHP2 and STAT3. This suggests that AMPK, a key cellular energy sensor, is an important intracellular link between metabolic and inflammatory pathways in liver.

PAPER IV

Summary of results

In *Paper IV* we investigated the impact of STK25 on skeletal muscle metabolism using the rat skeletal muscle cell line, L6. First, we measured the relative expression of *Stk25/STK25* mRNA expression in mouse, rat and human tissues and S*tk25/STK25* was broadly expressed in all tissue examined. Next, we determined the cellular localisation of endogenous STK25 in L6 myoblasts by immunofluorescent staining with STK25 showing a uniform cytoplasmatic distribution.

Lipid and glucose metabolism was investigated in L6 cells after repression of STK25 by siRNA transfection. A three- to four-fold depletion of STK25 was achieved in L6 cells by siRNA approach and it was stable over the period of observation (48, 96, 144 and 192 h post-transfection, *Paper IV,* ESM Fig. 1). We followed the expression as well as protein levels for genes known to be involved in fatty acid oxidation, glucose metabolism, as well as mitochondrial biogenesis. Most interestingly, we found the expression level for uncoupling protein 2 (*Ucp2*) and *Ucp3* to be increased in response to depletion of STK25. *Ucp2* was significantly increased at the first time point studied ($p<0.05$) and remained significantly increased over time ($p<0.01$, $p<0.01$, $p<0.05$, at 96, 144 and 192 h post-transfection, respectively). Markedly increased expression levels for *Ucp3* were seen at 144 h and 192 h post-transfection ($p<0.01$). Increased UCP3 was confirmed at protein level (1.65-fold increase at 192 h and 1.50-fold increase at 216 h post-transfection, ($p<0.05$)). However, we were not able to detect any UCP2 protein at any time point studied. Since UCP2 and UCP3 proteins are suggested to be involved in β-oxidation, we measured the rate of fatty acid oxidation after partial depletion of STK25 in L6 myoblasts. β-oxidation was increased approximately 20%, reaching the same level as measured for the positive control substance phenformin ($p<0.01$).

We also found the genes regulating glucose metabolism in skeletal muscle, *Glut1*, *Glut4* and *Hk2,* to be upregulated in response to STK25 depletion. Both *Glut1* and *Hk2* were significantly increased 96 h post-transfection and remained increased over the period of observation ($p<0.05$) while *Glut4* was found to be significantly induced 144 h post transfection ($p<0.05$). We confirmed an increased production of GLUT1, GLUT4, and HK2 at protein level ($p<0.05$; 144 h, 48 h and 96 h, 192 h post-transfection, respectively). Correspondingly, the glucose uptake rate, in response to insulin treatment, was increased approximately 50% after partial depletion of STK25 ($p<0.01$).

We also measured the activity of STK25 based on the phosphorylation level of Thr^{174} after L6 myoblasts were transiently transfected with pFLAG-r*Stk25* and treated with substances known to evoke different cellular stress responses. TNFα, but not IL-6,

significantly increased the phosphorylation level of STK25 ($p<0.05$). Both menadione and H_2O_2, known to induce oxidative stress, activated STK25 ($p<0.05$). Thapsigargin, a strong inducer of ER stress, or serum starvation did not affect the phosphorylation of STK25 (Fig. 8).

Interestingly, we found the expression level of *STK25* to be significantly increased in biopsies from skeletal muscle of T2D individuals ($p<0.05$). Based on our *in vitro* studies, we also quantified the expression of *HK2* and *UCP3* in these biopsies. *HK2* was decreased both in individuals with IGT ($p<0.05$) and T2D ($p<0.01$), whereas *UCP3* was significantly decreased in individuals with IGT ($p<0.05$).

Discussion

To our knowledge, this is the first study reporting STK25 to be involved in the regulation of glucose and lipid metabolism in skeletal muscle. In the experimental model used, partial depletion of STK25 in the rat myoblast cell line L6 leads to increased expression of genes involved in glucose and lipid metabolism - *Glut1*, *Glut4*, *Hk2* and *Ucp3*. Here, we show that increased level of GLUT1, GLUT4, HK2 and UCP3 correlates with increased insulin-stimulated glucose uptake and increased β–oxidation in L6 myoblasts. Increased mitochondrial lipid oxidation as well as increased clearance rate of glucose could be one way to improve metabolic dysfunctions in T2D and related complications.

Skeletal muscle is the major consumer of fatty acids as fuel and also accounts for approximately 70% of whole body insulin-stimulated glucose uptake. Therefore, an increased expression of the main glucose transporters *GLUT1* and *GLUT4* is expected to result in enhanced insulin sensitivity. In addition, after entering the muscle cells, glucose is phosphorylated by HK2 to form glucose-6-phosphate, and increased expression of *Hk2* is also expected to positively influence insulin-stimulated glucose metabolism (148-150).

UCPs are inner mitochondrial membrane transporters, with UCP1 being the first uncoupling protein to be identified. *UCP1* is expressed exclusively in brown adipose tissue (BAT) while *UCP2* is broadly distributed. *UCP3* is preferentially expressed in skeletal muscle and BAT (151; 152). The function of UCP3 and UCP2 has been re-evaluated and the importance of these two proteins in fatty acid metabolism has become more evident. Partial depletion of STK25 in L6 myoblasts results in increased expression of *Ucp2* and *Ucp3* mRNA as well as in increased UCP3 protein. However, we were unable to detect any UCP2 protein in L6 cells. Thereby, the physiological relevance of increased expression of *Ucp2* mRNA is unclear. It has been shown that the mRNA level for *Ucp3* is increased in rat skeletal muscle after fasting as a consequence of elevated free fatty acids (153). Furthermore, transgenic mice overexpressing UCP3 in skeletal muscle were found to be lean even though they eat

more than their wild-type littermate. In addition, these mice displayed reduced fasting glucose and insulin levels, which suggests improved insulin sensitivity (154). Interestingly, reduced UCP3 levels in skeletal muscle of mice reduce both AMPK activity as well as fatty acid oxidation, ultimately leading to decreased insulin sensitivity (155). MacLellan *et al*. reported that moderate overexpression of UCP3 in L6 myotubes increased fatty acid oxidation (156). It has also been shown that overexpression of UCP3 in L6 myotubes increases glucose uptake and GLUT4 translocation by activating a PI3K-dependent pathway (157).

There is a strong association between IR and lipid accumulation in nonadipose tissues, such as muscle and liver. Lipid accumulation could depend on increased fatty acid uptake, a decreased rate of fatty acid utilization, or a combination of both. Recent studies have reported that mitochondrial dysfunction could be a major factor contributing to muscle lipid accumulation and IR. It has been shown that individuals with T2D and IGT have reduced expression of key genes in oxidative metabolism and mitochondrial function in skeletal muscle (158; 159). Krook *et al*. reported that the mRNA level of *UCP3* in skeletal muscle biopsies was decreased in individuals with T2D (160). This was also shown at protein level by Schrauwenin *et al*. demonstrating that individuals with T2D had markedly lower UCP3 levels compared to control subjects (161). In our study we observed a reduced level of *UCP3* in skeletal muscle biopsies from individuals with IGT and a tendency toward reduced level in individuals with T2D, which coincides with elevated levels of *STK25* in individuals with T2D and lower levels of *HK2* in both individuals with T2D and IGT. In line with these results, a reduced gene expression of *HK2* has been reported in skeletal muscle from T2D patients (162).

The onset of diabetes is closely associated with oxidative stress due to hyperglycemia, which results in overproduction of oxygen free radicals. In general, cells could activate mechanisms that minimize the damage evoked by ROS or undergo cell death if the damage is too severe to repair. It has been shown that STK25 could be activated by oxidant stress in Ramos B cells or Madin-Darby canine kidney epithelial cells (112). This is in agreement with our results in L6 myoblast as STK25 was activated by menadione and H_2O_2, known to induce oxidative stress. In line with results presented by Pombo *et al*. (114), thapsigargin, an inducer of ER stress, was not able to activate STK25 in L6 myoblasts (Fig. 8).

In this study, relatively modest, although statistically significant increase in beta-oxidation and insulin-stimulated glucose uptake was seen in L6 cells. To evaluate the relevance of these findings on whole body level, the future focus is to further investigate the impact of STK25 *in vivo* by generating transgenic mice overexpressing STK25 as well as STK25 knockout mice. We have generated a transgenic mouse model overexpressing STK25 under an β-actin promoter. STK25 transgenic mice as

well as wild-type littermates have been challenged with HFD and early data from this study indicate that mice overexpressing STK25 have impaired insulin sensitivity and glucose tolerance. The studies in knockout mice, kindly provided by Prof. B. Howell, SUNY Upstate Medical University, Syracuse, NY, USA, are now ongoing.

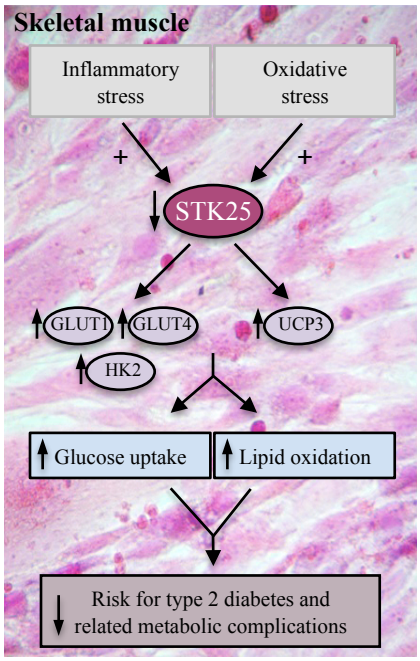

Figure 8. A proposed model of the role of STK25 in the regulation of lipid and glucose metabolism in skeletal muscle.

Conclusion

By partial depletion of STK25 in the rodent myoblast cell line L6 we demonstrate a modest but significantly increased lipid oxidation as well as insulin-stimulated glucose uptake, suggesting a role of STK25 as a negative regulator of glucose and lipid metabolism in skeletal muscle. Impaired glucose uptake and fatty acid metabolism by skeletal muscle is a hallmark of IR and, therefore, *S*TK25 could be an important new target to be evaluated for therapeutic intervention in T2D and related complications.

ACKNOWLEDGMENT

A lot of people have been involved and contributed in numerous ways to this work. I would like to express my gratitude to all of you and especially I would like to thank,

My main supervisor Margit Mahlapuu, who gave me the opportunity to explore the exciting research field of diabetes, for patient guidance, stimulating suggestions, useful critiques during my thesis writing and also in keeping my progress on schedule.

My co-supervisor Ulf Smith, for always being interested in the progress of my PhD project, giving me encouraging suggestions regarding following-up studies and also for providing laboratory facilities and equipments.

Aino Johansson, for being there for me when I started my PhD position, I am so happy to have had you as a colleague, co-author and friend.

Emmelie Cansby, for always being so enthusiastic and easy to collaborate with.

Christian Andersson, for always listing and supporting me with valuable suggestions regarding my research.

All co-authors, without you this thesis had never been written.

Birgit Gustafson, for always taking time answering all my questions and giving valuable advices and also for being my “officemate”.

Maria Pereira, for the opportunity to sharing valuable time with you in “baracken”.

Silvia Gogg, Ann Hammarstedt, Shahram Hedjazifar, Jenny Palming, Lachmi Jenndahl, Ida Sterner, Carina Blomgren, John Grünberg, Manoj Amrutkar, Lena Silberberg, and Jenny Hoffman, for always being helpful, answering questions, giving scientific advices and for many nice lunch breaks.

Gunilla Lindell, for administrative help and the pleasure to have you as a commuting friend.

Lisbeth Eriksson, for all administrative help during these years.

Björn Eliasson, for computer help.

All other past and present co-workers at the Lundberg Laboratory for Diabetes Research.

Finally, I would like to thank my family. Unfortunately, my parents are not here to share this moment with me, but I know that they had been very happy for me. Torbjörn, without your endless support during my “education periods” I had not been able to make it. I am also very happy for the support from my sons, Emil and Simon, and all nice family dinners we have had during this time. Kent, my brother for always being interested in the progress of my research.

Also thanks to all my friends how have always been there for me. Not least to my best and “oldest” friend Maria Fredricsson, for always being curious about my work and asking a lot of interesting question.

This work was supported by research grants from the Swedish Research Council, the Swedish Diabetes Association, the Swedish Agency for Innovation Systems, the Foundation for Scientific Studies of Diabetology, the Swedish Centre for Sports Research, the Swedish Foundation for Strategic Research, the Commission of the European Communities (Contract Nos. LSHM-CT-2004-005272 EXGENESIS and LSHM-CT-2004-512013 EUGENE2), the Swedish Medical Society, Stiftelsen Längmanska Kulturfonden, Johan and Jakob Söderbergs Foundation, Novo Nordisk Foundation, the European Foundation for the Study of Diabetes/Lilly research grant, the P. and A. Hedlunds Foundation, the L. Hierta Memorial Foundation, the Bergvall Foundation, the Diabetes Wellness Network Sweden, the Diabetesfonden, the Adlerbert Research Foundation, the Royal Society of Arts and Sciences Foundation and the Signe and Olof Wallenius Foundation.

REFERENCES

1. Shaw JE, Sicree RA, Zimmet PZ: Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract 2010;87:4-14
2. Rosenbloom AL, Joe JR, Young RS, Winter WE: Emerging epidemic of type 2 diabetes in youth. Diabetes Care 1999;22:345-354
3. Dabelea D, Pettitt DJ, Jones KL, Arslanian SA: Type 2 diabetes mellitus in minority children and adolescents. An emerging problem. Endocrinol Metab Clin North Am 1999;28:709-729, viii
4. Zhang P, Zhang X, Brown J, Vistisen D, Sicree R, Shaw J, Nichols G: Global healthcare expenditure on diabetes for 2010 and 2030. Diabetes Res Clin Pract 2010;87:293-301
5. Hu FB: Globalization of diabetes: the role of diet, lifestyle, and genes. Diabetes Care 2011;34:1249-1257
6. Fujimoto WY: The importance of insulin resistance in the pathogenesis of type 2 diabetes mellitus. Am J Med 2000;108 Suppl 6a:9S-14S
7. Weir GC, Bonner-Weir S: Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes 2004;53 Suppl 3:S16-21
8. Van Obberghen E, Baron V, Delahaye L, Emanuelli B, Filippa N, Giorgetti-Peraldi S, Lebrun P, Mothe-Satney I, Peraldi P, Rocchi S, Sawka-Verhelle D, Tartare-Deckert S, Giudicelli J: Surfing the insulin signaling web. Eur J Clin Invest 2001;31:966-977
9. Kido Y, Burks DJ, Withers D, Bruning JC, Kahn CR, White MF, Accili D: Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. J Clin Invest 2000;105:199-205
10. Saltiel AR, Kahn CR: Insulin signalling and the regulation of glucose and lipid metabolism. Nature 2001;414:799-806
11. Ahmad F, Cong LN, Stenson Holst L, Wang LM, Rahn Landstrom T, Pierce JH, Quon MJ, Degerman E, Manganiello VC: Cyclic nucleotide phosphodiesterase 3B is a downstream target of protein kinase B and may be involved in regulation of effects of protein kinase B on thymidine incorporation in FDCP2 cells. J Immunol 2000;164:4678-4688
12. Matsuzaki H, Daitoku H, Hatta M, Tanaka K, Fukamizu A: Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. Proc Natl Acad Sci U S A 2003;100:11285-11290
13. Lizcano JM, Alessi DR: The insulin signalling pathway. Curr Biol 2002;12:R236-238
14. Draznin B: Molecular mechanisms of insulin resistance: serine phosphorylation of insulin receptor substrate-1 and increased expression of p85alpha: the two sides of a coin. Diabetes 2006;55:2392-2397
15. Whitehead JP, Soos MA, Jackson R, Tasic V, Kocova M, O'Rahilly S: Multiple molecular mechanisms of insulin receptor dysfunction in a patient with Donohue syndrome. Diabetes 1998;47:1362-1364
16. George S, Rochford JJ, Wolfrum C, Gray SL, Schinner S, Wilson JC, Soos MA, Murgatroyd PR, Williams RM, Acerini CL, Dunger DB, Barford D, Umpleby AM, Wareham NJ, Davies HA, Schafer AJ, Stoffel M, O'Rahilly S, Barroso I: A family with

severe insulin resistance and diabetes due to a mutation in AKT2. Science 2004;304:1325-1328
17. Saini V: Molecular mechanisms of insulin resistance in type 2 diabetes mellitus. World J Diabetes 2010;1:68-75
18. Chavez JA, Knotts TA, Wang LP, Li G, Dobrowsky RT, Florant GL, Summers SA: A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. J Biol Chem 2003;278:10297-10303
19. Gregor MF, Hotamisligil GS: Inflammatory mechanisms in obesity. Annu Rev Immunol 2011;29:415-445
20. Wellen KE, Hotamisligil GS: Inflammation, stress, and diabetes. J Clin Invest 2005;115:1111-1119
21. Baffy G: Kupffer cells in non-alcoholic fatty liver disease: the emerging view. J Hepatol 2009;51:212-223
22. Solinas G, Karin M: JNK1 and IKKbeta: molecular links between obesity and metabolic dysfunction. FASEB J 2010;24:2596-2611
23. Nakamura T, Furuhashi M, Li P, Cao H, Tuncman G, Sonenberg N, Gorgun CZ, Hotamisligil GS: Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis. Cell 2010;140:338-348
24. Zick Y: Insulin resistance: a phosphorylation-based uncoupling of insulin signaling. Trends Cell Biol 2001;11:437-441
25. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F: Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 2003;374:1-20
26. Alonzi T, Maritano D, Gorgoni B, Rizzuto G, Libert C, Poli V: Essential role of STAT3 in the control of the acute-phase response as revealed by inducible gene inactivation [correction of activation] in the liver. Mol Cell Biol 2001;21:1621-1632
27. Lebrun P, Van Obberghen E: SOCS proteins causing trouble in insulin action. Acta Physiol (Oxf) 2008;192:29-36
28. Karlsson B, Knutsson A, Lindahl B: Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27,485 people. Occup Environ Med 2001;58:747-752
29. Rudic RD, McNamara P, Curtis AM, Boston RC, Panda S, Hogenesch JB, Fitzgerald GA: BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. PLoS Biol 2004;2:e377
30. Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS, Bass J: Obesity and metabolic syndrome in circadian Clock mutant mice. Science 2005;308:1043-1045
31. Narasimamurthy R, Hatori M, Nayak SK, Liu F, Panda S, Verma IM: Circadian clock protein cryptochrome regulates the expression of proinflammatory cytokines. Proc Natl Acad Sci U S A 2012;109:12662-12667
32. Matsumoto S, Basil J, Jetton AE, Lehman MN, Bittman EL: Regulation of the phase and period of circadian rhythms restored by suprachiasmatic transplants. J Biol Rhythms 1996;11:145-162

33. Ptitsyn AA, Zvonic S, Conrad SA, Scott LK, Mynatt RL, Gimble JM: Circadian clocks are resounding in peripheral tissues. PLoS Comput Biol 2006;2:e16
34. Zhang X, Dube TJ, Esser KA: Working around the clock: circadian rhythms and skeletal muscle. J Appl Physiol 2009;107:1647-1654
35. Zambon AC, McDearmon EL, Salomonis N, Vranizan KM, Johansen KL, Adey D, Takahashi JS, Schambelan M, Conklin BR: Time- and exercise-dependent gene regulation in human skeletal muscle. Genome Biol 2003;4:R61
36. Damiola F, Le Minh N, Preitner N, Kornmann B, Fleury-Olela F, Schibler U: Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev 2000;14:2950-2961
37. Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M: Entrainment of the circadian clock in the liver by feeding. Science 2001;291:490-493
38. Brown SA, Zumbrunn G, Fleury-Olela F, Preitner N, Schibler U: Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Curr Biol 2002;12:1574-1583
39. Rutter J, Reick M, Wu LC, McKnight SL: Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. Science 2001;293:510-514
40. Liu C, Li S, Liu T, Borjigin J, Lin JD: Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism. Nature 2007;447:477-481
41. Um JH, Yang S, Yamazaki S, Kang H, Viollet B, Foretz M, Chung JH: Activation of 5'-AMP-activated kinase with diabetes drug metformin induces casein kinase Iepsilon (CKIepsilon)-dependent degradation of clock protein mPer2. J Biol Chem 2007;282:20794-20798
42. Vieira E, Nilsson EC, Nerstedt A, Ormestad M, Long YC, Garcia-Roves PM, Zierath JR, Mahlapuu M: Relationship between AMPK and the transcriptional balance of clock-related genes in skeletal muscle. Am J Physiol Endocrinol Metab 2008;295:E1032-1037
43. Lamia KA, Sachdeva UM, DiTacchio L, Williams EC, Alvarez JG, Egan DF, Vasquez DS, Juguilon H, Panda S, Shaw RJ, Thompson CB, Evans RM: AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. Science 2009;326:437-440
44. Um JH, Pendergast JS, Springer DA, Foretz M, Viollet B, Brown A, Kim MK, Yamazaki S, Chung JH: AMPK regulates circadian rhythms in a tissue- and isoform-specific manner. PLoS One 2011;6:e18450
45. Barnea M, Haviv L, Gutman R, Chapnik N, Madar Z, Froy O: Metformin affects the circadian clock and metabolic rhythms in a tissue-specific manner. Biochim Biophys Acta 2012;1822:1796-1806
46. Asher G, Gatfield D, Stratmann M, Reinke H, Dibner C, Kreppel F, Mostoslavsky R, Alt FW, Schibler U: SIRT1 regulates circadian clock gene expression through PER2 deacetylation. Cell 2008;134:317-328
47. Nakahata Y, Kaluzova M, Grimaldi B, Sahar S, Hirayama J, Chen D, Guarente LP, Sassone-Corsi P: The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. Cell 2008;134:329-340
48. Nakahata Y, Sahar S, Astarita G, Kaluzova M, Sassone-Corsi P: Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1. Science 2009;324:654-657

49. Belden WJ, Dunlap JC: SIRT1 is a circadian deacetylase for core clock components. Cell 2008;134:212-214
50. Fu L, Lee CC: The circadian clock: pacemaker and tumour suppressor. Nat Rev Cancer 2003;3:350-361
51. Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB: Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 2002;109:307-320
52. Ueda HR, Hayashi S, Chen W, Sano M, Machida M, Shigeyoshi Y, Iino M, Hashimoto S: System-level identification of transcriptional circuits underlying mammalian circadian clocks. Nat Genet 2005;37:187-192
53. Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS, Weitz CJ: Role of the CLOCK protein in the mammalian circadian mechanism. Science 1998;280:1564-1569
54. Ko CH, Takahashi JS: Molecular components of the mammalian circadian clock. Hum Mol Genet 2006;15 Spec No 2:R271-277
55. Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U: The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 2002;110:251-260
56. Akashi M, Takumi T: The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock Bmal1. Nat Struct Mol Biol 2005;12:441-448
57. Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M, Kato Y, Honma K: Dec1 and Dec2 are regulators of the mammalian molecular clock. Nature 2002;419:841-844
58. Noshiro M, Usui E, Kawamoto T, Kubo H, Fujimoto K, Furukawa M, Honma S, Makishima M, Honma K, Kato Y: Multiple mechanisms regulate circadian expression of the gene for cholesterol 7alpha-hydroxylase (Cyp7a), a key enzyme in hepatic bile acid biosynthesis. J Biol Rhythms 2007;22:299-311
59. Ripperger JA, Schibler U: Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. Nat Genet 2006;38:369-374
60. Carling D, Zammit VA, Hardie DG: A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS Lett 1987;223:217-222
61. Merrill GF, Kurth EJ, Hardie DG, Winder WW: AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol 1997;273:E1107-1112
62. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ: Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. Diabetes 1998;47:1369-1373
63. Carling D, Thornton C, Woods A, Sanders MJ: AMP-activated protein kinase: new regulation, new roles? Biochem J 2012;445:11-27
64. Corton JM, Gillespie JG, Hardie DG: Role of the AMP-activated protein kinase in the cellular stress response. Curr Biol 1994;4:315-324

65. Carling D: The AMP-activated protein kinase cascade--a unifying system for energy control. Trends Biochem Sci 2004;29:18-24
66. Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Makela TP, Alessi DR, Hardie DG: Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J Biol 2003;2:28
67. Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M, Carling D: Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab 2005;2:21-33
68. Momcilovic M, Hong SP, Carlson M: Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. J Biol Chem 2006;281:25336-25343
69. Hardie DG, Hawley SA: AMP-activated protein kinase: the energy charge hypothesis revisited. Bioessays 2001;23:1112-1119
70. Davies SP, Helps NR, Cohen PT, Hardie DG: 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. FEBS Lett 1995;377:421-425
71. Carling D, Clarke PR, Zammit VA, Hardie DG: 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 1989;186:129-136
72. Suter M, Riek U, Tuerk R, Schlattner U, Wallimann T, Neumann D: Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. J Biol Chem 2006;281:32207-32216
73. Sanders MJ, Grondin PO, Hegarty BD, Snowden MA, Carling D: Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. Biochem J 2007;403:139-148
74. Xiao B, Sanders MJ, Underwood E, Heath R, Mayer FV, Carmena D, Jing C, Walker PA, Eccleston JF, Haire LF, Saiu P, Howell SA, Aasland R, Martin SR, Carling D, Gamblin SJ: Structure of mammalian AMPK and its regulation by ADP. Nature 2011;472:230-233
75. Hardie DG, Ross FA, Hawley SA: AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol 2012;13:251-262
76. Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, Winder WW: 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. Diabetes 1999;48:1667-1671
77. Treebak JT, Glund S, Deshmukh A, Klein DK, Long YC, Jensen TE, Jorgensen SB, Viollet B, Andersson L, Neumann D, Wallimann T, Richter EA, Chibalin AV, Zierath JR, Wojtaszewski JF: AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits. Diabetes 2006;55:2051-2058
78. Abbud W, Habinowski S, Zhang JZ, Kendrew J, Elkairi FS, Kemp BE, Witters LA, Ismail-Beigi F: Stimulation of AMP-activated protein kinase (AMPK) is associated with

enhancement of Glut1-mediated glucose transport. Arch Biochem Biophys 2000;380:347-352

79. Vavvas D, Apazidis A, Saha AK, Gamble J, Patel A, Kemp BE, Witters LA, Ruderman NB: Contraction-induced changes in acetyl-CoA carboxylase and 5'-AMP-activated kinase in skeletal muscle. J Biol Chem 1997;272:13255-13261
80. Jager S, Handschin C, St-Pierre J, Spiegelman BM: AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc Natl Acad Sci U S A 2007;104:12017-12022
81. Kahn BB, Alquier T, Carling D, Hardie DG: AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab 2005;1:15-25
82. Henin N, Vincent MF, Gruber HE, Van den Berghe G: Inhibition of fatty acid and cholesterol synthesis by stimulation of AMP-activated protein kinase. FASEB J 1995;9:541-546
83. Li Y, Xu S, Mihaylova MM, Zheng B, Hou X, Jiang B, Park O, Luo Z, Lefai E, Shyy JY, Gao B, Wierzbicki M, Verbeuren TJ, Shaw RJ, Cohen RA, Zang M: AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. Cell Metab 2011;13:376-388
84. Viollet B, Guigas B, Leclerc J, Hebrard S, Lantier L, Mounier R, Andreelli F, Foretz M: AMP-activated protein kinase in the regulation of hepatic energy metabolism: from physiology to therapeutic perspectives. Acta Physiol (Oxf) 2009;196:81-98
85. Koo SH, Flechner L, Qi L, Zhang X, Screaton RA, Jeffries S, Hedrick S, Xu W, Boussouar F, Brindle P, Takemori H, Montminy M: The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature 2005;437:1109-1111
86. Daval M, Foufelle F, Ferre P: Functions of AMP-activated protein kinase in adipose tissue. J Physiol 2006;574:55-62
87. Garton AJ, Campbell DG, Carling D, Hardie DG, Colbran RJ, Yeaman SJ: Phosphorylation of bovine hormone-sensitive lipase by the AMP-activated protein kinase. A possible antilipolytic mechanism. Eur J Biochem 1989;179:249-254
88. Gaidhu MP, Fediuc S, Anthony NM, So M, Mirpourian M, Perry RL, Ceddia RB: Prolonged AICAR-induced AMP-kinase activation promotes energy dissipation in white adipocytes: novel mechanisms integrating HSL and ATGL. J Lipid Res 2009;50:704-715
89. Corton JM, Gillespie JG, Hawley SA, Hardie DG: 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur J Biochem 1995;229:558-565
90. Owen MR, Doran E, Halestrap AP: Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J 2000;348 Pt 3:607-614
91. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA: An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). J Biol Chem 1995;270:12953-12956
92. Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I,

Matsuzawa Y: PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. Diabetes 2001;50:2094-2099

93. Brunmair B, Staniek K, Gras F, Scharf N, Althaym A, Clara R, Roden M, Gnaiger E, Nohl H, Waldhausl W, Furnsinn C: Thiazolidinediones, like metformin, inhibit respiratory complex I: a common mechanism contributing to their antidiabetic actions? Diabetes 2004;53:1052-1059
94. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, Kadowaki T: Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 2002;8:1288-1295
95. Sanders MJ, Ali ZS, Hegarty BD, Heath R, Snowden MA, Carling D: Defining the mechanism of activation of AMP-activated protein kinase by the small molecule A-769662, a member of the thienopyridone family. J Biol Chem 2007;282:32539-32548
96. Goransson O, McBride A, Hawley SA, Ross FA, Shpiro N, Foretz M, Viollet B, Hardie DG, Sakamoto K: Mechanism of action of A-769662, a valuable tool for activation of AMP-activated protein kinase. J Biol Chem 2007;282:32549-32560
97. Fogarty S, Hardie DG: Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. Biochim Biophys Acta 2010;1804:581-591
98. Nath N, Giri S, Prasad R, Salem ML, Singh AK, Singh I: 5-aminoimidazole-4-carboxamide ribonucleoside: a novel immunomodulator with therapeutic efficacy in experimental autoimmune encephalomyelitis. J Immunol 2005;175:566-574
99. Bai A, Ma AG, Yong M, Weiss CR, Ma Y, Guan Q, Bernstein CN, Peng Z: AMPK agonist downregulates innate and adaptive immune responses in TNBS-induced murine acute and relapsing colitis. Biochem Pharmacol 2010;80:1708-1717
100. Myerburg MM, King JD, Jr., Oyster NM, Fitch AC, Magill A, Baty CJ, Watkins SC, Kolls JK, Pilewski JM, Hallows KR: AMPK agonists ameliorate sodium and fluid transport and inflammation in cystic fibrosis airway epithelial cells. Am J Respir Cell Mol Biol 2010;42:676-684
101. Kim TB, Kim SY, Moon KA, Park CS, Jang MK, Yun ES, Cho YS, Moon HB, Lee KY: Five-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside attenuates poly (I:C)-induced airway inflammation in a murine model of asthma. Clin Exp Allergy 2007;37:1709-1719
102. Zhao X, Zmijewski JW, Lorne E, Liu G, Park YJ, Tsuruta Y, Abraham E: Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2008;295:L497-504
103. Sag D, Carling D, Stout RD, Suttles J: Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. J Immunol 2008;181:8633-8641
104. Cacicedo JM, Yagihashi N, Keaney JF, Jr., Ruderman NB, Ido Y: AMPK inhibits fatty acid-induced increases in NF-kappaB transactivation in cultured human umbilical vein endothelial cells. Biochem Biophys Res Commun 2004;324:1204-1209

105. Hattori Y, Suzuki K, Hattori S, Kasai K: Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells. Hypertension 2006;47:1183-1188
106. Bess E, Fisslthaler B, Fromel T, Fleming I: Nitric oxide-induced activation of the AMP-activated protein kinase alpha2 subunit attenuates IkappaB kinase activity and inflammatory responses in endothelial cells. PLoS One 2011;6:e20848
107. Zhang Y, Qiu J, Wang X, Zhang Y, Xia M: AMP-activated protein kinase suppresses endothelial cell inflammation through phosphorylation of transcriptional coactivator p300. Arterioscler Thromb Vasc Biol 2011;31:2897-2908
108. Yang Z, Kahn BB, Shi H, Xue BZ: Macrophage alpha1 AMP-activated protein kinase (alpha1AMPK) antagonizes fatty acid-induced inflammation through SIRT1. J Biol Chem 2010;285:19051-19059
109. Green CJ, Pedersen M, Pedersen BK, Scheele C: Elevated NF-kappaB activation is conserved in human myocytes cultured from obese type 2 diabetic patients and attenuated by AMP-activated protein kinase. Diabetes 2011;60:2810-2819
110. Kim YD, Kim YH, Cho YM, Kim DK, Ahn SW, Lee JM, Chanda D, Shong M, Lee CH, Choi HS: Metformin ameliorates IL-6-induced hepatic insulin resistance via induction of orphan nuclear receptor small heterodimer partner (SHP) in mouse models. Diabetologia 2012;55:1482-1494
111. Dan I, Watanabe NM, Kusumi A: The Ste20 group kinases as regulators of MAP kinase cascades. Trends Cell Biol 2001;11:220-230
112. Pombo CM, Bonventre JV, Molnar A, Kyriakis J, Force T: Activation of a human Ste20-like kinase by oxidant stress defines a novel stress response pathway. EMBO J 1996;15:4537-4546
113. Osada S, Izawa M, Saito R, Mizuno K, Suzuki A, Hirai S, Ohno S: YSK1, a novel mammalian protein kinase structurally related to Ste20 and SPS1, but is not involved in the known MAPK pathways. Oncogene 1997;14:2047-2057
114. Pombo CM, Tsujita T, Kyriakis JM, Bonventre JV, Force T: Activation of the Ste20-like oxidant stress response kinase-1 during the initial stages of chemical anoxia-induced necrotic cell death. Requirement for dual inputs of oxidant stress and increased cytosolic [Ca2+]. J Biol Chem 1997;272:29372-29379
115. Preisinger C, Short B, De Corte V, Bruyneel E, Haas A, Kopajtich R, Gettemans J, Barr FA: YSK1 is activated by the Golgi matrix protein GM130 and plays a role in cell migration through its substrate 14-3-3zeta. J Cell Biol 2004;164:1009-1020
116. Fidalgo M, Fraile M, Pires A, Force T, Pombo C, Zalvide J: CCM3/PDCD10 stabilizes GCKIII proteins to promote Golgi assembly and cell orientation. J Cell Sci 2010;123:1274-1284
117. Voss K, Stahl S, Schleider E, Ullrich S, Nickel J, Mueller TD, Felbor U: CCM3 interacts with CCM2 indicating common pathogenesis for cerebral cavernous malformations. Neurogenetics 2007;8:249-256
118. Matsuki T, Matthews RT, Cooper JA, van der Brug MP, Cookson MR, Hardy JA, Olson EC, Howell BW: Reelin and stk25 have opposing roles in neuronal polarization and dendritic Golgi deployment. Cell 2010;143:826-836

119. Nogueira E, Fidalgo M, Molnar A, Kyriakis J, Force T, Zalvide J, Pombo CM: SOK1 translocates from the Golgi to the nucleus upon chemical anoxia and induces apoptotic cell death. J Biol Chem 2008;283:16248-16258
120. Zhou J, Shao Z, Kerkela R, Ichijo H, Muslin AJ, Pombo C, Force T: Serine 58 of 14-3-3zeta is a molecular switch regulating ASK1 and oxidant stress-induced cell death. Mol Cell Biol 2009;29:4167-4176
121. Ling P, Lu TJ, Yuan CJ, Lai MD: Biosignaling of mammalian Ste20-related kinases. Cell Signal 2008;20:1237-1247
122. Barnes BR, Marklund S, Steiler TL, Walter M, Hjalm G, Amarger V, Mahlapuu M, Leng Y, Johansson C, Galuska D, Lindgren K, Abrink M, Stapleton D, Zierath JR, Andersson L: The 5'-AMP-activated protein kinase gamma3 isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle. J Biol Chem 2004;279:38441-38447
123. Mahlapuu M, Johansson C, Lindgren K, Hjalm G, Barnes BR, Krook A, Zierath JR, Andersson L, Marklund S: Expression profiling of the gamma-subunit isoforms of AMP-activated protein kinase suggests a major role for gamma3 in white skeletal muscle. Am J Physiol Endocrinol Metab 2004;286:E194-200
124. Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB: Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 1979;282:615-616
125. Baumann H, Won KA, Jahreis GP: Human hepatocyte-stimulating factor-III and interleukin-6 are structurally and immunologically distinct but regulate the production of the same acute phase plasma proteins. J Biol Chem 1989;264:8046-8051
126. Klier FG, Schubert D, Heinemann S: The ultrastructural differentiation of the clonal myogenic cell line L6 in normal and high K+ medium. Dev Biol 1977;57:440-449
127. Wilkening S, Stahl F, Bader A: Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. Drug Metab Dispos 2003;31:1035-1042
128. Kawamata T, Tomari Y: Making RISC. Trends Biochem Sci 2010;35:368-376
129. Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HR: Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn 2005;5:209-219
130. Romero-Calvo I, Ocon B, Martinez-Moya P, Suarez MD, Zarzuelo A, Martinez-Augustin O, de Medina FS: Reversible Ponceau staining as a loading control alternative to actin in Western blots. Anal Biochem 2010;401:318-320
131. Hansen PA, Gulve EA, Holloszy JO: Suitability of 2-deoxyglucose for in vitro measurement of glucose transport activity in skeletal muscle. J Appl Physiol 1994;76:979-985
132. Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, Auwerx J: AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature 2009;458:1056-1060
133. Froy O: The circadian clock and metabolism. Clin Sci (Lond) 2011;120:65-72
134. Caton PW, Kieswich J, Yaqoob MM, Holness MJ, Sugden MC: Metformin opposes impaired AMPK and SIRT1 function and deleterious changes in core clock protein

expression in white adipose tissue of genetically-obese db/db mice. Diabetes Obes Metab 2011;13:1097-1104

135. Hatori M, Vollmers C, Zarrinpar A, DiTacchio L, Bushong EA, Gill S, Leblanc M, Chaix A, Joens M, Fitzpatrick JA, Ellisman MH, Panda S: Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. Cell Metab 2012;15:848-860
136. Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L: Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J 1998;334 (Pt 2):297-314
137. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G: Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am J Physiol Endocrinol Metab 2001;280:E745-751
138. Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW: Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. J Clin Endocrinol Metab 1997;82:4196-4200
139. Eder K, Baffy N, Falus A, Fulop AK: The major inflammatory mediator interleukin-6 and obesity. Inflamm Res 2009;58:727-736
140. Panesar N, Tolman K, Mazuski JE: Endotoxin stimulates hepatocyte interleukin-6 production. J Surg Res 1999;85:251-258
141. Nagaraju K, Raben N, Merritt G, Loeffler L, Kirk K, Plotz P: A variety of cytokines and immunologically relevant surface molecules are expressed by normal human skeletal muscle cells under proinflammatory stimuli. Clin Exp Immunol 1998;113:407-414
142. Pedersen BK, Febbraio MA: Muscle as an endocrine organ: focus on muscle-derived interleukin-6. Physiol Rev 2008;88:1379-1406
143. Kelly M, Keller C, Avilucea PR, Keller P, Luo Z, Xiang X, Giralt M, Hidalgo J, Saha AK, Pedersen BK, Ruderman NB: AMPK activity is diminished in tissues of IL-6 knockout mice: the effect of exercise. Biochem Biophys Res Commun 2004;320:449-454
144. Bode JG, Albrecht U, Haussinger D, Heinrich PC, Schaper F: Hepatic acute phase proteins--regulation by IL-6- and IL-1-type cytokines involving STAT3 and its crosstalk with NF-kappaB-dependent signaling. Eur J Cell Biol 2012;91:496-505
145. Ramadoss P, Unger-Smith NE, Lam FS, Hollenberg AN: STAT3 targets the regulatory regions of gluconeogenic genes in vivo. Mol Endocrinol 2009;23:827-837
146. Mashili F, Chibalin AV, Krook A, Zierath JR: Constitutive STAT3 Phosphorylation Contributes to Skeletal Muscle Insulin Resistance in Type 2 Diabetes. Diabetes 2012;
147. Serrano-Marco L, Barroso E, El Kochairi I, Palomer X, Michalik L, Wahli W, Vazquez-Carrera M: The peroxisome proliferator-activated receptor (PPAR) beta/delta agonist GW501516 inhibits IL-6-induced signal transducer and activator of transcription 3 (STAT3) activation and insulin resistance in human liver cells. Diabetologia 2012;55:743-751
148. Halseth AE, Bracy DP, Wasserman DH: Overexpression of hexokinase II increases insulinand exercise-stimulated muscle glucose uptake in vivo. Am J Physiol 1999;276:E70-77

149. Fueger PT, Heikkinen S, Bracy DP, Malabanan CM, Pencek RR, Laakso M, Wasserman DH: Hexokinase II partial knockout impairs exercise-stimulated glucose uptake in oxidative muscles of mice. Am J Physiol Endocrinol Metab 2003;285:E958-963
150. Fueger PT, Bracy DP, Malabanan CM, Pencek RR, Granner DK, Wasserman DH: Hexokinase II overexpression improves exercise-stimulated but not insulin-stimulated muscle glucose uptake in high-fat-fed C57BL/6J mice. Diabetes 2004;53:306-314
151. Vidal-Puig A, Solanes G, Grujic D, Flier JS, Lowell BB: UCP3: an uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue. Biochem Biophys Res Commun 1997;235:79-82
152. Boss O, Samec S, Paoloni-Giacobino A, Rossier C, Dulloo A, Seydoux J, Muzzin P, Giacobino JP: Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. FEBS Lett 1997;408:39-42
153. Weigle DS, Selfridge LE, Schwartz MW, Seeley RJ, Cummings DE, Havel PJ, Kuijper JL, BeltrandelRio H: Elevated free fatty acids induce uncoupling protein 3 expression in muscle: a potential explanation for the effect of fasting. Diabetes 1998;47:298-302
154. Clapham JC, Arch JR, Chapman H, Haynes A, Lister C, Moore GB, Piercy V, Carter SA, Lehner I, Smith SA, Beeley LJ, Godden RJ, Herrity N, Skehel M, Changani KK, Hockings PD, Reid DG, Squires SM, Hatcher J, Trail B, Latcham J, Rastan S, Harper AJ, Cadenas S, Buckingham JA, Brand MD, Abuin A: Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. Nature 2000;406:415-418
155. Senese R, Valli V, Moreno M, Lombardi A, Busiello RA, Cioffi F, Silvestri E, Goglia F, Lanni A, de Lange P: Uncoupling protein 3 expression levels influence insulin sensitivity, fatty acid oxidation, and related signaling pathways. Pflugers Arch 2011;461:153-164
156. MacLellan JD, Gerrits MF, Gowing A, Smith PJ, Wheeler MB, Harper ME: Physiological increases in uncoupling protein 3 augment fatty acid oxidation and decrease reactive oxygen species production without uncoupling respiration in muscle cells. Diabetes 2005;54:2343-2350
157. Huppertz C, Fischer BM, Kim YB, Kotani K, Vidal-Puig A, Slieker LJ, Sloop KW, Lowell BB, Kahn BB: Uncoupling protein 3 (UCP3) stimulates glucose uptake in muscle cells through a phosphoinositide 3-kinase-dependent mechanism. J Biol Chem 2001;276:12520-12529
158. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, Groop LC: PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 2003;34:267-273
159. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ: Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci U S A 2003;100:8466-8471

160. Krook A, Digby J, O'Rahilly S, Zierath JR, Wallberg-Henriksson H: Uncoupling protein 3 is reduced in skeletal muscle of NIDDM patients. Diabetes 1998;47:1528-1531
161. Schrauwen P, Hesselink MK, Blaak EE, Borghouts LB, Schaart G, Saris WH, Keizer HA: Uncoupling protein 3 content is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes 2001;50:2870-2873
162. Vestergaard H, Bjorbaek C, Hansen T, Larsen FS, Granner DK, Pedersen O: Impaired activity and gene expression of hexokinase II in muscle from non-insulin-dependent diabetes mellitus patients. J Clin Invest 1995;96:2639-2645