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Sernevi Säll, Johanna

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LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Salt-inducible kinases in adipose tissue

Salt-inducible kinases in adipose tissue

Johanna Sernevi Säll

LUND UNIVERSITY

DOCTORAL DISSERTATION
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Margit Mahlapuu
Sahlgrenska Academy
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Abstract

Obesity, insulin resistance and type 2 diabetes (T2D) are metabolic diseases that increase rapidly in the modern society. The underlying mechanisms are complex and involve both environmental and genetic factors. Adipose tissue is an important organ for maintaining whole body energy homeostasis. In response to nutrient overload (as observed in obesity), adipocytes become hypertrophic and dysfunctional, which contribute to the development of systemic insulin resistance. AMP-activated protein kinase (AMPK) is described as a master regulator of whole body energy homeostasis. The salt-inducible kinases (SIKs) are related to AMPK. SIK2 displays abundant expression in adipose tissue and has been reported to be increased in adipose tissue in obese diabetic (*db/db*) mice, suggesting that SIK2 potentially is involved in the development of obesity and T2D. Taken together, this makes the SIKs, and SIK2 in particular, interesting to study in adipose tissue in the context of obesity and insulin resistance. This thesis has addressed the expression, regulation and biological role of SIKs, in particular SIK2, in adipose tissue. So far, studies on SIKs in adipose tissue have exclusively been performed in rodents. This thesis focuses on studying the role of SIKs in humans.

We demonstrate that the transcriptional regulators CRTC2, CRTC3 and HDAC4 are direct downstream substrates of SIK2 in adipocytes. Moreover, we demonstrate that the expression of SIK2 and SIK3 is markedly downregulated in adipose tissue from obese or insulin-resistant individuals. Furthermore, the expression of SIK2 and SIK3 in adipose tissue is regulated in response to weight change and inflammation (TNF-α). SIKs are involved in promoting glucose uptake in adipocytes and the underlying mechanism(s) involves direct, and positive, effects on the insulin signalling pathway. We also identify a novel regulatory pathway of SIK2 in adipocytes through insulin-induced phosphorylation at Thr484. From a functional aspect, insulin stimulation appears to be important to increase SIK2 protein stability. Taken together, our data suggest that insulin resistance might be a causal factor underlying the downregulation of SIK2 in human adipose tissue. Given these findings, SIK2 might provide an attractive therapeutic target for the treatment of metabolic diseases in the future. Importantly, our findings on SIK2 expression in human obesity and insulin resistance are in contrast to what was previously identified in mice, and demonstrates that interspecies differences exist with regard to the regulation of SIK2 in metabolic disease. Furthermore, this emphasise the importance to study SIKs in human adipocytes.

Key words
salt-inducible kinase, SIK, SIK2, adipose tissue, adipocytes, insulin signalling, glucose uptake, obesity, insulin resistance

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Salt-inducible kinases in adipose tissue

Johanna Sernevi Säll

Protein Phosphorylation Research Group
Department of Experimental Medical Science
Faculty of Medicine

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Till mina hjärtan (Christoffer, Silja och dig vi väntar på)

ABSTRACT

Obesity, insulin resistance and type 2 diabetes (T2D) are metabolic diseases that increase rapidly in the modern society. The underlying mechanisms are complex and involve both environmental and genetic factors. Adipose tissue is an important organ for maintaining whole body energy homeostasis. In response to nutrient overload (as observed in obesity), adipocytes become hypertrophic and dysfunctional, which contribute to the development of systemic insulin resistance. AMP-activated protein kinase (AMPK) is described as a master regulator of whole body energy homeostasis. The salt-inducible kinases (SIKs) are related to AMPK. SIK2 displays abundant expression in adipose tissue and has been reported to be increased in adipose tissue in obese diabetic (*db/db*) mice, suggesting that SIK2 is potentially involved in the development of obesity and T2D. Taken together, this makes the SIKs, and SIK2 in particular, interesting to study in adipose tissue in the context of obesity and insulin resistance. This thesis has addressed the expression, regulation and biological role of SIKs, in particular SIK2, in adipose tissue. So far, studies on SIKs in adipose tissue have exclusively been performed in rodents. This thesis focuses on studying the role of SIKs in humans.

We demonstrate that the transcriptional regulators CRTC2, CRTC3 and HDAC4 are direct downstream substrates of SIK2 in adipocytes. Moreover, we demonstrate that the expression of SIK2 and SIK3 is markedly downregulated in adipose tissue from obese or insulin-resistant individuals. Furthermore, the expression of SIK2 and SIK3 in adipose tissue is regulated in response to weight change and inflammation (TNF-α). SIKs are involved in promoting glucose uptake in adipocytes and the underlying mechanism(s) involves direct, and positive, effects on the insulin signalling pathway. We also identify a novel regulatory pathway of SIK2 in adipocytes through insulin-induced phosphorylation at Thr484. From a functional aspect, insulin stimulation appears to be important to increase SIK2 protein stability. Taken together, our data suggest that insulin resistance might be a causal factor underlying the downregulation of SIK2 in human adipose tissue. Given these findings, SIK2 might provide an attractive therapeutic target for the treatment of metabolic diseases in the future. Importantly, our findings on SIK2 expression in human obesity and insulin resistance are in contrast to what was previously identified in mice, and demonstrates that interspecies differences exist with regard to the regulation of SIK2

in metabolic disease. Furthermore, this emphasise the importance to study SIKs in human adipocytes.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Fetma och typ 2 diabetes är stora folksjukdomar som ökar i rasande takt världen över. En gemensam nämnare för dessa sjukdomar är att de vävnader i kroppen som reglerar ämnesomsättningen, t.ex. fettvävnaden, förlorar känsligheten för hormonet insulin så att det inte längre fungerar normalt. Detta kallas insulinresistens och i min forskning undersöker jag hur kroppens fettvävnad fungerar för att förstå vad som kan gå fel när sjukdomar som fetma och typ 2 diabetes utvecklas.

Kroppens byggstenar kallas celler och varje vävnad består av miljontals celler med olika specialistkompetenser. Den vävnad jag är särskilt intresserad av i min forskning är fettväven som, kanske inte förvånande, till största delen består av fettceller. Om man sedan förstorar ytterligare och tittar inuti varje enskild cell så finns där mängder med proteiner som möjliggör att cellen får sitt särskilda utseende och sin speciella funktion.

Fettväven är viktig för att reglera energibalansen i kroppen. Fettcellerna är unika på så vis att de är specialiserade på att lagra fett, som kan användas för att ge andra organ i kroppen energi vid behov. Fettcellerna är fyllda till bredden av fett och allt annat innehåll knuffas ut mot det omslutande höljet.

Efter en måltid stiger blodsockret och hormonet insulin utsöndras från bukspottkörteln. Insulinet transporteras via blodet till bl. a. fettväven där det passar som nyckeln i ett lås i ett mottagarprotein på cellens yta. Att insulinet binder in aktiverar en kaskadreaktion inuti cellerna. Denna kaskadreaktion är cellernas sätt att kommunicera för att bestämma vad de olika proteinerna inuti cellen ska göra just för tillfället, t. ex. släppa in socker från blodet.

Kommunikationen inuti cellerna styrs av en stor familj proteiner som kallas *kinaser* och de överför budskap genom en process som kallas *proteinfosforylering*. Man skulle kunna likna dessa proteiner vid brevbärare som tar emot signaler utifrån, sorterar dem och sedan skickar dem vidare till rätt ställe inuti cellen. Dessa signaler är livsviktiga och krävs för att vi varje sekund ska kunna andas, tänka och röra oss. Det är när något går fel i denna kommunikation och budskapet inte når fram, eller om det förändras, som sjukdomar utvecklas.

Den forskning jag bedriver är inriktad på att studera signalvägar i fettceller av betydelse för uppkomsten av fetma och typ 2 diabetes, med fokus kring kinaset *SIK2* (eng. salt-inducible kinase 2). SIK2 finns i alla kroppens vävnader men det finns särskilt mycket av SIK2 i fettväven. Tidigare studier har visat att mängden SIK2 är förändrad hos försöksdjur med fetma och typ 2 diabetes, vilket ger en ledtråd om att SIK2 sannolikt spelar en viktig roll för uppkomsten av dessa sjukdomar även i människor. Men trots att det finns mer SIK2 i fettväven än någon annanstans i kroppen så är det relativt okänt vad SIK2 har för funktion i denna vävnad. Det är därför både relevant och viktigt att ta reda på mer om funktionen av SIK2 i fettväv ur både ett fetma- och diabetesperspektiv.

Målet för min forskning är att undersöka vilka signaler utanför cellen som kan påverka SIK2 och vilka andra proteiner inuti fettcellen som SIK2 i sin tur kan kontrollera och vad detta får för effekt på cellens funktioner, såsom upptag av socker. Ytterligare ett mål är att kartlägga om mängden SIK2 i fettväv skiljer sig mellan feta och smala personer, samt personer med insulinresistens.

Våra studier visar att SIK2 kan påverkas på molekylär nivå av hormon som är viktiga för ämnesomsättningen i kroppen och att det är involverat i att främja processer med betydelse för fettvävens funktion såsom upptag av socker. Vi har också sett att personer med fetma eller insulinresistens har sänkta nivåer av SIK2 i sin fettväv. Med min forskning hoppas jag kunna bidra med en liten pusselbit till gåtan bakom fetma och typ 2 diabetes för att vi i framtiden ska bli bättre på att förebygga eller bota dessa sjukdomar.

LIST OF PAPERS

Papers included in this thesis

I. **SIK2 regulates CRTCs, HDAC4 and glucose uptake in adipocytes.** Emma Henriksson, Johanna Säll, Amélie Gormand, Sebastian Wasserstrom, Nicholas A. Morrice, Andreas M. Fritzen, Marc Foretz, David G. Campbell, Kei Sakamoto, Mikael Ekelund, Eva Degerman, Karin G. Stenkula, Olga Göransson. *Journal of Cell Science* (2015), 128(3): 472-486.

II. **Salt-inducible kinase 2 and -3 are downregulated in adipose tissue from obese or insulin-resistant individuals: implications for insulin signalling and glucose uptake in human adipocytes.** Johanna Säll, Annie ML. Pettersson, Christel Björk, Emma Henriksson, Sebastian Wasserstrom, Wilhelm Linder, Yuedan Zhou, Ola Hansson, Daniel P. Andersson, Mikael Ekelund, Eva Degerman, Karin G. Stenkula, Jurga Laurencikiene, Olga Göransson. *Diabetologia* (2017), 60(2): 314-323.

III. **Insulin induces Thr484 phosphorylation and stabilization of SIK2 in adipocytes.** Johanna Säll, Annie ML. Pettersson, Wilhelm Linder, Mikael Ekelund, Jurga Laurencikiene, Eva Degerman, Karin G. Stenkula, Olga Göransson. *Manuscript.*

IV. **Salt-inducible kinases promote glucose uptake by stimulating insulin signalling in human adipocytes.** Johanna Säll, Andreas M. Fritzen, Maria Lindahl, Wilhelm Linder, Anna Warvsten, Eva Degerman, Bente Kiens, Karin G. Stenkula, Olga Göransson. *Manuscript.*

Papers not included in this thesis

I. **The antimicrobial peptide LL-37 alters human osteoblast Ca^{2+} handling and induces Ca^{2+}-independent apoptosis.** Johanna Säll, Martin Carlsson, Olof Gidlöf, Anders Holm, Johan Humlén, Jenny Öhman, Daniel Svensson, Bengt-Olof Nilsson, Daniel Jönsson. *Journal of Innate Immunity* (2013), 5(3): 290-300.

II. **Stretch-sensitive down-regulation of the miR-144/451 cluster in vascular smooth muscle and its role in AMP-activated protein kinase signaling.** Karolina M. Turczyńska, Anirban Bhattachariya, Johanna Säll, Olga Göransson, Karl Swärd, Per Hellstrand, Sebastian Albinsson. *PLoS One* (2013), 8(5): e65135.

III. **Rosiglitazone drives cavin-2/SDPR expression in adipocytes in a CEBPα-dependent manner.** Björn Hansson, Catarina Rippe, Dorota Kotowska, Sebastian Wasserstrom, Johanna Säll, Olga Göransson, Karl Swärd, Karin G. Stenkula. *PLoS One* (2017), 12(3): e0173412.

ABBREVIATIONS

ACC	acetyl-CoA carboxylase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AMPK-RK	AMPK-related kinase
AP-1	activator protein 1
AS160	Akt substrate of 160 kDa
ATGL	adipose triglyceride lipase
BRSK	brain-specific kinase
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CaMKK	CaMK kinase
cAMP	cyclic AMP
CAR	coxsackievirus and adenovirus receptor
C/EBP	CCAAT/enhancer-binding protein
ChoRE	carbohydrate-responsive element
ChREBP	ChoRE-binding protein
CHX	cycloheximide
CoA	coenzyme A
CREB	cAMP-responsive element-binding protein
CRTC	CREB-regulated transcription coactivator
DNA	deoxyribonucleic acid
ENCODE	Encyclopedia of DNA Elements
ER	endoplasmic reticulum
FA	fatty acid

FAHFAs	FA esters of hydroxy FAs
FAS	FA synthase
FOXO	forkhead box O
G6Pase	glucose-6-phosphatase
GLUT	glucose transporter
HA	hemagglutinin
HAT	histone acetyltransferase
HDAC	histone deacetylase
HFD	high-fat diet
HOMA-IR	homeostatic model assessment of insulin resistance
HSL	hormone sensitive lipase
IBMX	isobutylmethylxanthine
IKK	inhibitor of κB kinase
IL-	interleukin-
IR	insulin receptor
IRS	insulin receptor substrate
JNK	c-Jun N-terminal kinase
KO	knockout
LKB1	liver kinase B1
MARK	MAP/microtubule affinity-regulating kinase
MCP-1	monocyte chemoattractant protein 1
MEF2	myocyte enhancer factor 2
MELK	maternal embryonic leucine zipper kinase
miR-	microRNA
MGL	monoacylglycerol lipase
mRNA	messenger RNA
mTORC2	mammalian target of rapamycin complex 2
NF-κB	nuclear factor κB
PDE	phosphodiesterase

PDK1	3-phosphoinositide-dependent protein kinase 1
PEPCK	phosphoenolpyruvate carboxykinase
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP_3	phosphatidylinositol-(3,4,5)-trisphosphate
PKA	protein kinase A
PKB/Akt	protein kinase B
PKC	protein kinase C
PP	protein phosphatase
PPARγ	peroxisome proliferator-activated receptor γ
PTB	phosphotyrosine binding
qRT-PCR	real-time reverse transcription polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
scWAT	subcutaneous WAT
SH2	Src homology 2
SIK	salt-inducible kinase
siRNA	small/short interfering RNA
SOCS	suppressor of cytokine signalling
SREBP	sterol regulatory element-binding protein
Ser/Thr	serine/threonine
SVF	stromal-vascular fraction
T2D	type 2 diabetes
TAG	triacylglycerol
TGF-β	transforming growth factor β
TNF-α	tumour necrosis factor-α
TRB3	tribbles homolog 3
UCP1	uncoupling protein 1
UPR	unfolded protein response

vWAT	visceral WAT
WAT	white adipose tissue
WT	wild type

GENERAL INTRODUCTION

As a consequence of the lifestyle changes we experience in the urbanised world, obesity is a rapidly increasing health problem worldwide. The World Health Organization estimated that in 2014, more than 1 in 10 were obese (BMI>30 kg/m^2) [1]. Moreover, obesity is a major risk factor for developing secondary conditions such as type 2 diabetes (T2D). Obesity is characterised by the excessive accumulation of body fat in the adipose tissue. Adipose tissue is a multifunctional organ with the principal purpose to store and release energy depending on the current nutrient supply. Adipose tissue also functions as an endocrine organ and releases hormones, adipokines, that are important for regulating whole-body energy homeostasis. Adipocyte function is tightly regulated by the actions of the opposing hormones insulin and catecholamines. After a meal, insulin promote the uptake of glucose and fatty acids in adipocytes and their subsequent storage as triacylglycerols (TAGs) in a large intracellular reservoir. When energy demand increases, TAGs are broken down and the resulting glycerol and fatty acids are transported to peripheral tissues to be used as energy substrate. In states of chronic nutrient overload the adipocytes becomes enlarged (hypertrophic) due to the shifted balance in lipid synthesis vs. breakdown. Following the development of hypertrophic adipocytes is a sequence of events that involves the infiltration of inflammatory cells in the adipose tissue and an altered secretion of inflammatory mediators and adipokines. Together this contributes to the manifestation of insulin resistance and a dysfunctional adipose tissue. In insulin resistance, a tissue loses its ability to respond in a normal manner to insulin. In adipose tissue, insulin is not only important to promote the synthesis of lipids but also to shut down the breakdown of TAGs to ensure that opposing pathways are not operating at the same time. Consequently, in states of insulin resistance the breakdown of TAGs to glycerol and fatty acids cannot be properly inhibited, which contributes to increased circulating plasma lipid levels. When excess lipids no longer can be stored in adipose tissue, they accumulate in other metabolic tissues (e.g. muscle, liver) that are not dedicated for this purpose. The storage of lipids in non-adipose tissues impairs their function and progressively causes systemic insulin resistance. In summary, adipose tissue plays an important role in promoting the development of inflammation and insulin resistance [2-4].

AMP-activated protein kinase (AMPK) is often described as a master metabolic regulator and plays important roles in the regulation of whole-body energy homeostasis. AMPK is also a therapeutic target for the pharmacological treatment of metabolic diseases such as T2D [5-8]. In 2002, a family of 12 kinases related to AMPK was discovered – the AMPK-related kinases [9]. They share sequence similarities to AMPK in their kinase domains, suggesting that they regulate similar substrates and cellular processes. The subfamily of salt-inducible kinases (SIKs) consists of three members – SIK1, SIK2 and SIK3 [10]. Of these, SIK2 is enriched in adipose tissue [11, 12] and has been reported to be upregulated in adipose tissue of genetically obese diabetic mice and to modulate the insulin signalling pathway [11]. Therefore, SIK2 is of particular interest to study in adipose tissue in the context of obesity and T2D. Relative AMPK, the biological roles of SIKs have not been extensively studied, but given their relationship to the master metabolic regulator AMPK, SIKs have been implicated to play an important role in the regulation of metabolic processes [13]. This thesis focuses on studying the expression, regulation and biological functions of salt-inducible kinases (SIKs), in particular SIK2, in adipose tissue. Compared to previous work on SIKs in adipose tissue, my research has been focused on understanding the role of SIKs in human adipose tissue. An increased understanding of the signalling pathways that underlie a dysfunctional adipose tissue is important for the development of better preventions and/or treatments for metabolic diseases in the future.

SCIENTIFIC BACKGROUND

Adipose tissue

The white adipose tissue (WAT) is an organ that serves several different functions in the body – primarily it acts as an energy reservoir that is dynamically regulated in response to whole-body energy status. In addition, adipose tissue functions as padding around the organs and as an insulating layer [2]. In recent years, adipose tissue has also been recognised as an important endocrine organ that secretes various hormones, termed adipokines [14]. The most well characterised adipokines include leptin [15] and adiponectin [16], which are involved in regulating appetite and systemic insulin sensitivity. Apart from the WAT, an adipose tissue depot that has gained large interest due to its positive role in energy expenditure is the brown adipose tissue (BAT) [17].

Adipose tissue physiology

The majority of the adipose tissue volume is comprised by adipocytes. In addition to adipocytes, the adipose tissue contains a stromal-vascular fraction (SVF) that consists of adipocyte progenitors (pre-adipocytes and stem cells), fibroblasts, endothelial and immune cells (macrophages) [18]. The adipose tissue is distributed in specific depots in the body, each depot having different metabolic properties [18]. In humans, the main adipose tissue depots are the subcutaneous (scWAT), and the visceral (vWAT) adipose tissues [2]. Adipocytes develop from progenitors in a process called adipogenesis upon the activation of a complex transcriptional network controlled by the transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα) [4]. Adipose tissue is constantly being regenerated by the turnover of adipocytes that generally have a lifespan of ≈8 years [19]. The total number of adipocytes and pre-adipocytes is determined during adolescence [20] and remain constant even during obesity [19].

The white adipocyte has a unique morphology with a large unilocular lipid droplet covering almost the entire intracellular space [18]. The cytoplasm including the nucleus and other organelles is forced to the periphery of the cell [3]. Adipocytes are cells specialised in storing lipids. By responding to hormonal signals (insulin and catecholamines), adipose tissue alternates between storing glucose and fatty acids

(FAs) as triacylglycerol (TAG) (lipogenesis), or releasing glycerol and FAs to the circulation to be used as an energy substrate for peripheral tissues (lipolysis) [2]. The surface of the lipid droplet is covered with structural proteins and metabolic enzymes that are involved in the regulation of lipid metabolism [3].

Hormonal regulation of adipose tissue function

Adipose tissue function is controlled by the opposing actions of the hormones insulin and catecholamines (adrenaline and noradrenaline) [3]. A schematic summary is shown in Figure 1. In the fed state, plasma glucose levels increase and trigger the release of insulin from β-cells in the pancreas. Insulin bind to the insulin receptor (IR) located at the cell surface, resulting in receptor autophosphorylation and activation of its intrinsic tyrosine kinase activity. The adaptor protein insulin receptor substrate 1 (IRS1) binds to the phosphorylated tyrosine residues in the IR via pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains. Consequently, tyrosine phosphorylation of IRS1 by IR enables recruitment of phosphatidylinositol 3-kinase (PI3K) that binds IRS1 via its Src homology 2 (SH2) domain. PI3K is a dimer of a regulatory p85 subunit and a catalytic p110 subunit. PI3K generates phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) that attract protein kinase B (PKB/Akt) and 3-phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane. PDK1 phosphorylates PKB/Akt at Thr308. For full activation PKB/Akt also requires phosphorylation at Ser473 by mammalian target of rapamycin complex 2 (mTORC2) [21, 22]. PKB/Akt act as a central node in the insulin signalling pathway, and is a key regulator of multiple downstream targets including Akt substrate of 160 kDa (AS160, also known as TBC1D4) [23]. PKB/Akt-phosphorylation of AS160 at Thr642 inhibits its activity and results in a derepression of glucose transporter 4 (GLUT4) translocation to the plasma membrane, and an increased uptake of glucose [24]. PKB/Akt also phosphorylates and inhibits glycogen synthase kinase 3 (GSK3) causing increased glycogen synthesis, and inhibits the transcription factor forkhead box O1 (FOXO1) to decrease hepatic gluconeogenesis [21, 22]. The majority of glucose taken up by the adipocyte is metabolised to glycerol and used together with dietary fatty acids (FAs) to synthesise TAGs through esterification. Glucose can also be used for *de novo* FA synthesis from acetyl-coenzyme A (CoA) [3]. The processes of *de novo* FA synthesis and TAG synthesis are generally called lipogenesis. The expression of lipogenic genes – acetyl-CoA carboxylase (ACC) and FA synthase (FAS) – is controlled via carbohydrate-response element-binding protein (ChREBP) in adipocytes [2].

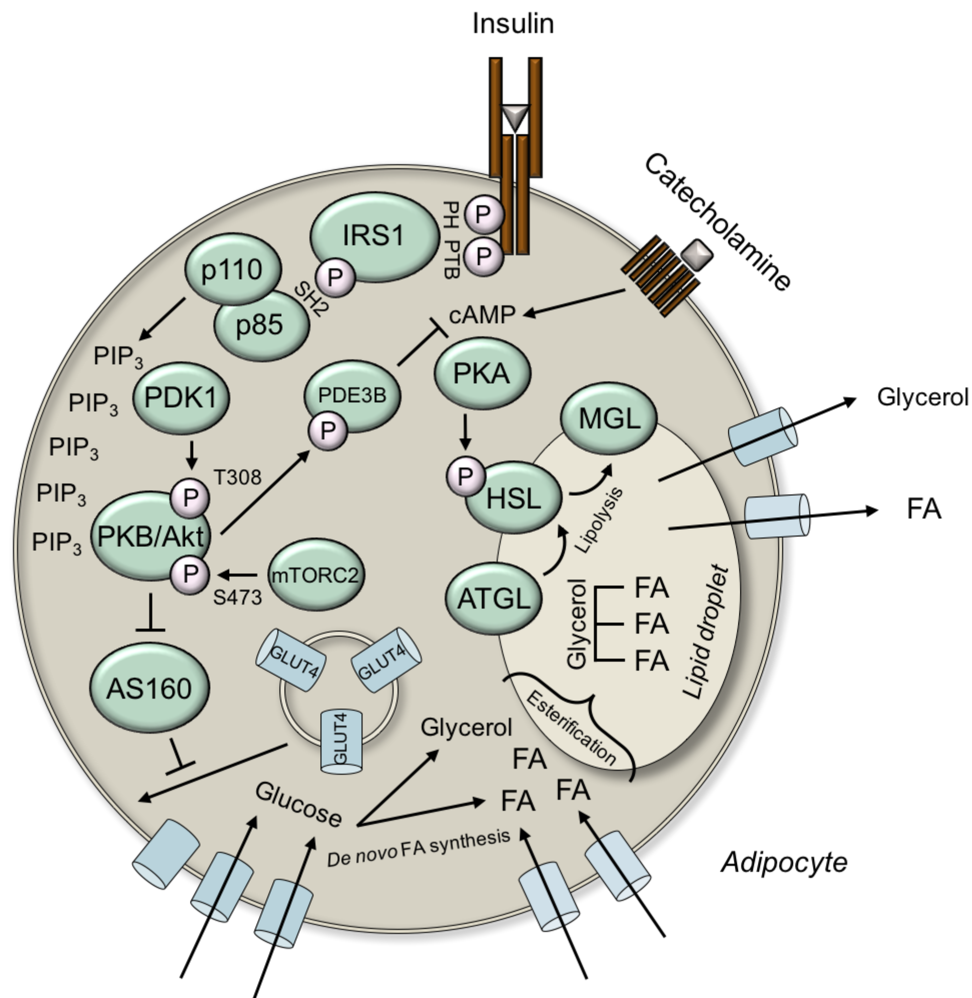

Figure 1. Schematic illustrating the hormonal regulation of adipocyte metabolism.
In the fed state, circulating insulin bind to insulin receptors, which subsequently initiates a signalling cascade that ultimately results in the translocation of GLUT4 to the plasma membrane and an increased glucose uptake. Glucose is used for the synthesis of glycerol or FA. In addition, dietary FAs are taken up from the circulation. Glycerol and FAs are esterified to form TAGs that are stored in the lipid droplet. In the fasting state, catecholamines bind to β-adrenergic receptors to induce the production of cAMP. In turn, cAMP activates PKA that phosphorylates and activates HSL. TAGs are hydrolysed in three consequtive steps to generate FAs and glycerol that are transported into the bloodstream for peripheral tissues to use as an energy source. The cross talk between anabolic and catabolic glucose and lipid metabolism is accomplished by the insulin-induced activation of PDE3B through phosphorylation by PKB/Akt. PDE3B hydrolyses cAMP to inhibit the activation of PKA and lipolytic pathways when nutrients are abundant.

In the fasted state, when energy levels are low, catecholamines are secreted by the adrenal glands. Catecholamines bind to β-adrenergic receptors to induce production of the second messenger cAMP that activates PKA. In turn, PKA phosphorylates perilipin and hormone sensitive lipase (HSL). Activated lipases associate to the surface of the lipid droplet. Adipose triglyceride lipase (ATGL) initiate the breakdown of

lipids (lipolysis), followed by the actions of HSL and monoacylglycerol lipase (MGL) to release FAs and glycerol to the circulation [2, 3]. The balance between the activation of the insulin signalling and cAMP/PKA-pathways is controlled by phosphodiesterase 3B (PDE3B). PKB/Akt-induced phosphorylation and activation of PDE3B results in hydrolysis of cAMP, and subsequently an inhibition of lipase activities (anti-lipolysis) [25]. This cross talk between two opposing hormonal pathways enables a balance in adipocyte lipid and glucose metabolism depending on whole-body energy status.

Obesity and metabolic diseases

Obesity is defined as having a body mass index (BMI) equal to or above 30 kg/m^2. The World Health Organisation (WHO) estimated that in 2014, over 600 million adults (13 % of the population) worldwide were obese [1]. Since then, this number has unquestionably continued to increase. Although certain predisposing genetic factors may exist, obesity is most commonly a result of excess intake of nutrients in combination with a sedentary lifestyle [26]. Obesity is a major risk factor for developing secondary conditions, particularly insulin resistance, type 2 diabetes (T2D) and cardiovascular diseases [4]. Obesity, insulin resistance and T2D are characterised by a state of chronic or low-grade inflammation, mainly localised to the adipose tissue, that is believed to be triggered by the metabolic overload [27, 28].

Adipose tissue expansion in obesity

WAT is a flexible tissue that is capable of dynamic expansion in response to the current nutrient supply. Expansion primarily occurs through adipocyte enlargement (hypertrophy), and successively by increasing adipocyte differentiation from the resident pool of progenitors (hyperplasia) [2, 4]. However, in response to long-term nutrient overload and the subsequent hypertrophy, adipocytes can eventually reach limits that prevent further expansion [2, 3]. Failure of WAT to accommodate the increased energy supply leads to lipid storage in other metabolic tissues (e.g. muscle, liver) that are not designated for this purpose, a process called ectopic lipid deposition (lipotoxicity) [4]. In addition, a dysfunctional adipose tissue is associated with remodelling of the cytoskeleton and extracellular matrix, and hypoxia [3]. Both scWAT and vWAT expand during the development of obesity, but the dysregulation of vWAT is considered more pathogenic in the context of metabolic diseases [18].

Mechanisms underlying insulin resistance

To date, the exact pathogenesis underlying the systemic insulin resistance commonly associated with T2D has not been fully understood and several pathways likely contribute. However, adipose tissue dysfunction plays an important role in the development of obesity-induced insulin resistance in peripheral tissues.

Adipose tissue expansion is associated with an altered adipocyte secretory profile, involving an elevation of pro-inflammatory cytokines (e.g. tumour necrosis factor-α [TNF-α], interleukin-1β [IL-1β], IL-6 and monocyte chemoattractant protein 1 [MCP-1]) and a reduction of anti-inflammatory mediators (eg. adiponectin and branched FA esters of hydroxy FAs [FAHFAs]) [4, 29]. TNF-α was the first cytokine identified to be overexpressed in adipose tissue in obesity [30, 31], and demonstrated to play a direct role in the modulation of insulin sensitivity [32, 33]. The inflammatory milieu recruits macrophages that infiltrate adipose tissue, and further increase the release of pro-inflammatory cytokines [34, 35]. Moreover, the increased metabolic demand during energy excess pose an increased stress on certain organelles, such as the endoplasmic reticulum (ER) and mitochondria. These stressors lead to an activation of the unfolded protein response (UPR) and the generation of reactive oxygen species (ROS) [3]. Adipose tissue dysfunction interferes with adipocyte lipid metabolism resulting in a release of FAs into the circulation and ectopic lipid deposition that in turn contribute to the progressive development of peripheral insulin resistance in obesity [3, 29].

The combinatory actions of inflammation, ER and oxidative stress, and an accumulation of lipid metabolites are in turn linked to the activation of signalling pathways that have been implicated in the development of systemic insulin resistance, downstream of c-Jun N-terminal kinase (JNK), inhibitor of κB kinase (IKK) and protein kinase C (PKC) isoforms [22, 28]. The pathways underlying insulin resistance are believed to converge at the level of IRS in the insulin signalling pathway [22, 28]. In addition to tyrosine phosphorylation by IR, IRS is subjected to multiple phosphorylations at serine/threonine (Ser/Thr) residues that result in both positive and negative effects on the downstream signalling pathway. The aberrant activation of JNK, IKK and PKCs in response to metabolic stress cause inhibitory Ser/Thr phosphorylation of IRS that disrupts its ability to transduce signals and elicit a cellular response [22]. Additionally, suppressor of cytokine signalling (SOCS) proteins have been described to interfere with IRS1 tyrosine phosphorylation and promote proteasomal degradation [36, 37].

LKB1/AMPK-signalling pathways

AMP-activated protein kinase (AMPK) is an evolutionary conserved Ser/Thr kinase that displays ubiquitous expression [5]. It acts as a sensor of cellular energy status (AMP/ADP:ATP ratio) and regulates metabolic processes to maintain cellular energy homeostasis. In a metabolic context, AMPK balances the utilisation and storage of glucose and lipids. By responding to hormonal and nutrient signals, AMPK also coordinates the control of energy homeostasis at the whole-body level [6, 8].

AMPK is a heterotrimeric protein complex consisting of a catalytic α-subunit, and regulatory β- and γ-subunits. AMPK is activated in response to energy stress by AMP or ADP binding to the γ-subunit, which results in a net increase in the phosphorylation at Thr172 in the activation loop, located in the kinase domain of the α-subunit [6, 8]. Thr172 is phosphorylated by the constitutively active upstream kinase liver kinase B1 (LKB1) [38]. This phosphorylation enable full activation of AMPK. AMP/ADP binding also cause a conformational change that protects the phosphorylation at Thr172 from the actions of protein phosphatases [6, 8].

Role of AMPK in the regulation of metabolism

The major target tissues for AMPK action are the liver, muscle and adipose tissue. Activation of AMPK promotes the metabolic flux through ATP-generating (catabolic) processes while suppressing ATP-consuming (anabolic) processes in order to restore the intracellular energy balance. AMPK mediates both acute effects by direct phosphorylation of metabolic enzymes and more long-term effects by phosphorylation of transcriptional regulators that in turn regulate gene expression. Acute effects of AMPK activation include increased glucose uptake and FA oxidation in muscle tissues, and inhibition of FA synthesis in the liver and adipose tissue. Sustained activation of AMPK contributes to metabolic reprogramming by inhibiting the transcriptional programmes regulating lipogenesis and gluconeogenesis in the liver. Additionally, AMPK inhibits protein synthesis and inflammation, and promotes mitochondrial biogenesis and autophagy [6, 8, 39].

AMPK-related kinases (AMPK-RKs)

In 2002, Manning et al made a detailed characterisation of all protein kinases encoded within the human genome, termed the human kinome [9]. With this classification a family related to the catalytic α-subunit of AMPK, consisting of an additional 12 kinases, was identified – the AMPK-related kinases (AMPK-RKs) (Figure 2). The AMPK-RKs share sequence similarities to AMPK in their highly conserved N-terminal kinase domain indicating similar substrate specificity. Later, Lizcano et al (2004) described that all these kinases (except MELK) are activated by the same upstream LKB1 complex [38]. The sequences of AMPK-RKs also contain a ubiquitin-associated (UBA) domain that is believed to be important for stabilising the kinase domain of the proteins to promote the activating phosphorylation by LKB1 [40] (except for MAP/microtubule affinity-regulating kinase 1 [MARK1] and MARK2 [41]). The AMPK-RKs are distinct from AMPK in that they consist of a single subunit and cannot be activated by AMP [10].

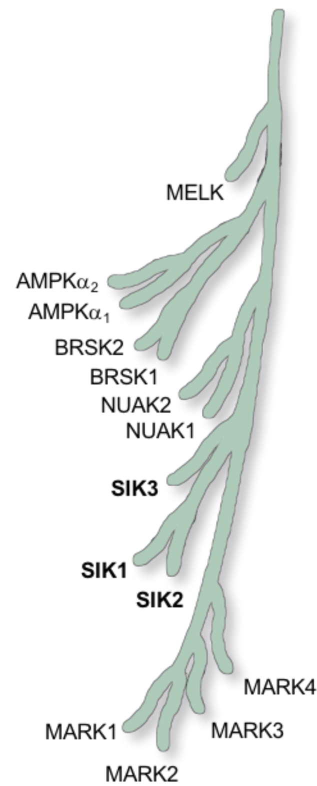

Figure 2. The AMPK-family of kinases.
Branch of the human kinome presented by Manning et al (2002) illustrating the AMPK-family of kinases [9]. The AMPK-family consist of AMPK catalytic α_1 and α_2-subunits, MELK (maternal embryonic leucine zipper kinase), BRSK1-2 (brain-specific kinase), NUAK1 and NUAK2 (ARK5 and SNARK), SIK1-3 (salt-inducible kinase) and MARK1-4 (MAP/microtubule affinity-regulating kinase).

Lessons from LKB1-knockout models

Global knockout of the upstream AMPK/SIK-activating kinase LKB1 in mice results in embryonic lethality demonstrating the importance of LKB1-signalling for the control of fundamental physiological processes [42]. To date, numerous LKB1 and AMPK knockout models have been described. The most interesting from an adipose tissue perspective are the models with an adipose-tissue specific deletion. Two different adipose tissue-specific LKB1 knockout mouse models have been described [43, 44]. From those reports, it is clear that LKB1-signalling is involved in the regulation of adipogenesis and adipocyte differentiation but the results point in different directions. However, studies in isolated cells suggest that LKB1-signalling pathways negatively regulates these processes [45]. Recently, the first inducible global LKB1 knockout mouse was described [46] and this model made it possible to study

the role of LKB1-signalling in adult mice. In this setting, LKB1 is required to maintain body weight and adipose tissue mass [46]. LKB1 deletion typically results in robust/severe phenotypes, most likely due to the simultaneous disruption of several downstream signalling pathways. Importantly, LKB1 regulates multiple downstream pathways and knockout of LKB1 alone does not distinguish which downstream signalling pathway that actually contributes to the observed phenotypes. Since AMPK is the major downstream target of LKB1 and has been well characterised, many of the metabolic effects of LKB1 deletion was first attributed to AMPK. However, later studies using conditional AMPK knockout models, and the development of more specific AMPK activators, have revealed that AMPK is in fact dispensable for some of the metabolic changes in response to LKB1 deletion indicating that the AMPK-RKs instead play an important role for certain effects.

Salt-inducible kinases (SIKs)

The subfamily of salt-inducible kinases (SIKs) consist of three related proteins – SIK1, SIK2 and SIK3 (Figure 2). SIK1 was first identified to be induced in the adrenal glands of rats fed a high-salt diet, hence the name "salt-inducible" [49]. Later, SIK2 and SIK3 were identified by sequence homology [50, 51]. The gene encoding *SIK1* is located on chromosome 21, and the genes for *SIK2* and *SIK3* on chromosome 11 [50]. Figure 3 depicts a schematic summary of human SIK protein sequences including conserved domains, and reported phosphorylation sites (described in greater detail below).

The N-terminal regions containing the kinase domain are highly conserved between the three SIK isoforms, whereas the C-terminal regions differ in length and display large sequence variability. This is assumed to give each kinase characteristic regulatory functions. In addition to the kinase and UBA domains, SIK1 also contains a region rich in arginine and lysine residues (RK) [52]. SIKs are Ser/Thr protein kinases that are involved in the regulation of a wide range of cellular processes, discussed further below [10, 13, 51].

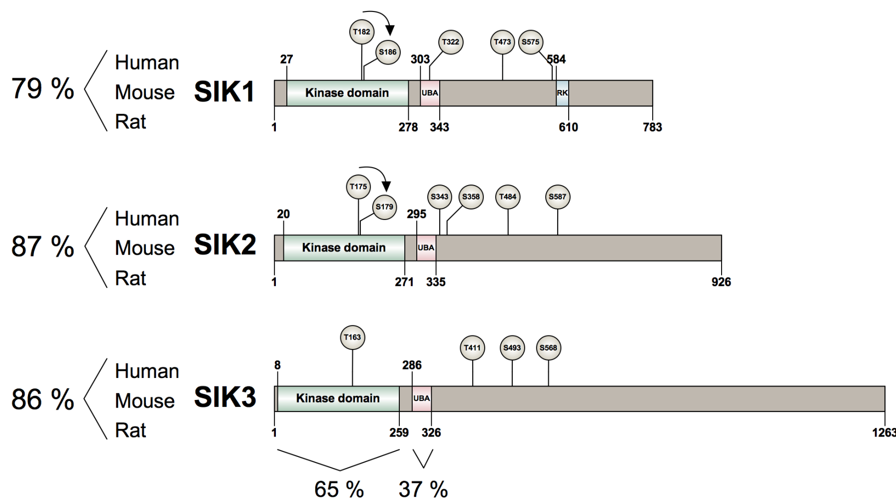

Figure 3. Schematic summary of SIK protein sequences.
Schematic depicting full-length human SIK protein sequences including kinase (green) and UBA (pink) domains. Each respective isoform is highly conserved between human, mouse and rat, and display 79 % (SIK1), 87 % (SIK2) and 86 % (SIK3) similarity when comparing the full protein sequences. Similarities between SIK1, SIK2 and SIK3 kinase and UBA domains are 65 % and 37 %, respectively. SIK1 also contains an RK-rich region (blue). Reported phosphorylation sites are illustrated (beige circles). Sequence numbering for human SIKs is shown. Protein sequences were created using the software "Illustrator for Biological Sequences" (IBS) [53].

Tissue distribution of SIKs

In the first report on mammalian SIK2, Horike et al compared the expression of *Sik1* and *Sik2* mRNA in various mouse tissues and observed that *Sik1* displayed highest expression in the adrenal gland, whereas *Sik2* was enriched in adipose tissues (WAT and BAT) [11]. In WAT of genetically diabetic *db/db* mice, the expression of SIK2 protein and SIK2 *in vitro* kinase activity were upregulated (*Sik2* mRNA was unchanged) [11]. Later, Du et al (2008) compared the levels of SIK2 protein in a panel of mouse tissues and found that SIK2 was most abundant in murine adipose tissues also on protein level [12]. In that report, the expression of *SIK2* mRNA was compared in human tissues for the first time. *SIK2* mRNA displayed a similar expression pattern in humans as in mice, with the highest levels in WAT. The expression of *Sik3* mRNA was reported to be ubiquitous [50, 51]. Recently, Darling et al (2017) determined SIK kinase activity in a range of mouse tissues and observed

that SIK1, SIK2 and SIK3 activity was present in all the tissues analysed. The total SIK activity was highest in adipose tissue, with SIK2 being the dominant isoform in this tissue. SIK1 activity was generally low in the tissues analysed, and SIK3 showed the highest activity in the brain [54]. The Genotype-Tissue Expression (GTEx) project has determined the global RNA expression profile in multiple human tissues [55]. In this database, *SIK1*, *SIK2* and *SIK3* display highest expression in the skin, WAT and brain, respectively. Together, these data demonstrate that the different SIK isoforms have a unique tissue distribution pattern, potentially reflecting their relative importance in specific tissues. However, in most cases the expression was only determined on mRNA level and one should bear in mind that the mRNA expression does not always translate directly to the protein levels. Nonetheless, there is no doubt that the expression of SIK2 is highest in adipose tissue since all mentioned reports demonstrate its dominance in this tissue, both on mRNA and protein level, as well as in mouse and in human. The reason for the varying conclusions about the expression patterns of SIK1 and SIK3 between different reports might be explained by the fact that different panels of tissues were analysed in the different studies. Also, species-specific differences in the expression might exist.

Regulation of SIKs by extracellular signals

In addition to the intrinsic kinase activity that is controlled by LKB1 through phosphorylation of a threonine residue in the activation loop (Thr175 in SIK2), the catalytic and cellular activities of SIKs can be further regulated by additional phosphorylations by other kinases. Several of these phosphorylation sites are conserved between the three SIK isoforms but might be regulated by different stimuli and generate different functional output depending on the tissue. In our work we have chosen to distinguish between the catalytic and cellular activity of a kinase. The catalytic activity is a quantifiable number that measures the amount of phosphate that is transferred to a substrate by the kinase per unit of time *in vitro*. The cellular activity reflects the ability of a kinase to phosphorylate a substrate in intact cells and can, in addition to changes in the intrinsic kinase activity, be affected by protein-protein interactions, intracellular localisation or protein stability that in turn can affect the ability of a kinase to act on downstream substrates. Studies have shown that phosphorylation of SIK2 in response to extracellular stimuli does not necessarily alter the catalytic activity but instead influence the cellular activity of SIK2 [56, 57]. Thus, it is important to keep in mind that a phosphorylation that does not result in a change in the *in vitro* kinase activity might still be functionally important to control its cellular activity.

As discussed above, insulin and catecholamines are hormones that are essential for adipose tissue function. The SIKs have been demonstrated to be phosphorylated in

response to both these stimuli in different tissues. However, the regulation by PKA has been more extensively studied. Figure 4 depicts a schematic summary of reported SIK phosphorylation sites, and the proposed upstream kinases.

Unbiased analyses of cAMP/PKA-induced phosphorylation sites have been performed for SIK2 [56-58] and SIK3 [58, 59]. SIK2 is phosphorylated by PKA at several residues (Ser343, Ser358, Thr484 and Ser587) in response to cAMP-elevation in adipocytes [56] and hepatocytes [57], without altering the intrinsic catalytic activity. Mackenzie et al (2013) reported that SIK2 was phosphorylated by PKA at Ser343 in macrophages [58]. Phosphorylated peptides for Ser358 and Ser587 were also detected, but did not display any induction in response to cAMP/PKA in these cells. SIK2 was also shown to be phosphorylated at Ser358 upon cAMP/PKA-activation in β-cells and islets [60]. Residues Ser343 and Ser358 in SIK2 are not conserved in SIK1 or SIK3.

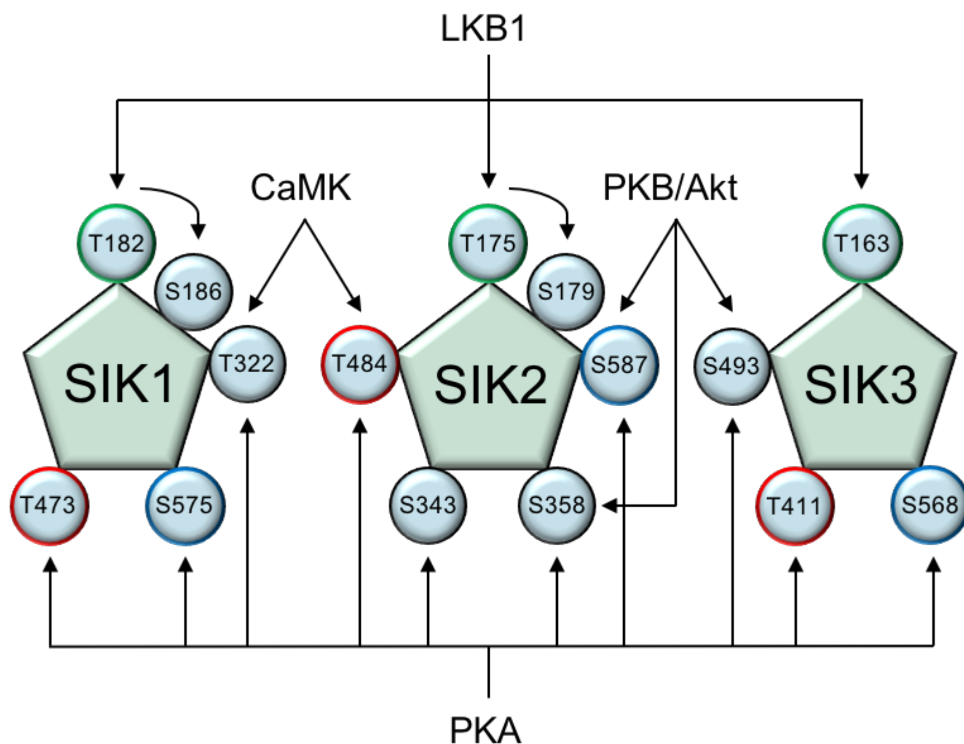

Figure 4. Reported SIK phosphorylation sites.
Schematic depicting reported SIK phosphorylation sites and the respective upstream kinases. Phosphorylation sites conserved between SIK1, SIK2 and SIK3 are illustrated with different coloured circles (green, red and blue). Sequence numbering for these sites in human, mouse and rat is shown in Figure 5.

SIK3 was phosphorylated on multiple residues to varying degrees (the major being Thr411, Ser493, Ser568) in response to cAMP/PKA-activation. Several of the identified sites likely contribute to the PKA-induced effects as simultaneous mutation of four sites (Thr163, Thr411, Ser493 and Ser568) was required to completely ablate PKA-phosphorylation. Thr411 and Ser493 in SIK3 was also found to be induced by cAMP-elevation in hepatocytes [61]. In β-cells, SIK3 was shown to be phosphorylated at PKA-consensus site in response cAMP/PKA-activation [60]. A recent study reported that SIK3 was phosphorylated at Ser493 following stimulation with NaCl and/or inflammatory cytokine IL-17 in breast cancer cells, and the upstream mechanism was suggested to involve the mTORC2/PKB/Akt-pathway [62]. Phosphorylated peptides corresponding to Thr411 and Ser493 was also detected in macrophages, but they were not responsive to cAMP-induction in these cells [58]. The Ser493 residue in SIK3 is not conserved in SIK1 or SIK2. A translation of sequence numbering for conserved sites between species is presented in Figure 5.

	Human	Mouse	Rat
SIK1	182	182	182
SIK2	175	175	175
SIK3	163	163	221

	Human	Mouse	Rat
SIK1	473	475	475
SIK2	484	484	484
SIK3	411	411	469

	Human	Mouse	Rat
SIK1	575	577	577
SIK2	587	587	586
SIK3	568	616	674

Figure 5. Conserved SIK phosphorylation sites.
Translation of residue numbering for conserved phosphorylation sites in human, mouse and rat SIK1, SIK2 and SIK3.

The RK-rich region in SIK1 functions as a nuclear import signal that determines the predominantly nuclear localisation of SIK1 in resting cells [52]. PKA-induced phosphorylation of Ser575, localised just outside this region, disrupts the nuclear localisation of SIK1 and cause a translocation to the cytoplasm in adrenal cells [52, 63]. Thus, the phosphorylation of SIK1 at Ser575 is important for controlling nucleocytoplasmic shuttling of SIK1. The equivalent phosphorylation site is conserved also in SIK2 (Ser587) and SIK3 (Ser568) and is similarly regulated upon cAMP/PKA-activation [56, 59]. However, SIK2 and SIK3 lack the RK-rich region and display essentially cytoplasmic localisation, independently of the phosphorylation status at Ser587 and Ser568, respectively [11, 56, 59]. In 3T3-L1 adipocytes, PKA-induced phosphorylation of SIK2 at Ser587 was associated with an increased activity of CRE-promoters [11]. Interestingly, a similar regulation of SIK2 was identified in brown adipocytes, but in response to insulin instead [64]. This suggest that SIK2, like SIK1, is involved in the control of CREB-dependent gene transcription and that the regulation of Ser587 display tissue-specific differences. On the other hand, SIK2 was reported to be phosphorylated by PKB/Akt at Ser358 in response to insulin stimulation of hepatocytes [65]. In these cells, insulin-induced phosphorylation increased the catalytic activity of SIK2. Kuser-Abali et al (2013) also described

activation of SIK2 by insulin in retinal glial cells [66]. Furthermore, insulin did not alter either phosphorylation or catalytic activity of SIK2 [56, 57] or SIK3 [59] in adipocytes and hepatocytes. In *Drosophila*, activation of the analogous insulin signalling pathway was demonstrated to increase PKB/Akt-dependent phosphorylation of SIK3, but the specific site regulated was not identified [67]. Together, these data suggest that the regulation of SIK isoforms by insulin with regards to phosphorylation and catalytic activity displays tissue-specific effects, and needs further investigation. The regulation of SIK1 in response to insulin has to our knowledge not been studied.

In SIK1, Thr475 (corresponding to human Thr473) is phosphorylated upon activation of cAMP/PKA-signalling in myocytes, and phosphorylation at this site was reported to increase SIK1 protein stability [68]. The equivalent site in SIK2 (Thr484), phosphorylated by CaMKI/IV in response to ischemia and increases in intracellular Ca^{2+}, was instead reported to target SIK2 for proteasomal degradation in neurons [69]. One report also described that SIK1 is phosphorylated at Thr322 by CaMK1 *in vitro* [70]. Additionally, SIK1 and SIK2 are capable of autophosphorylation at the residue located at the +4-position relative the threonine phosphorylated by LKB1 [71].

As previously mentioned, phosphorylations induced by extracellular stimuli does not necessarily alter the catalytic activity of SIKs, but might instead regulate other aspects of SIK function such as their association with 14-3-3 proteins [56, 59, 72]. 14-3-3 are scaffolding proteins that generally induce conformational changes and alter the cellular activity of the interacting protein [73]. Several of the phosphorylated residues in the SIKs have been reported to constitute binding sites for 14-3-3 proteins. SIK1 and SIK3 bind 14-3-3 directly to the threonine phosphorylated by LKB1, resulting in an intracellular relocalisation and increased catalytic activity towards downstream targets [72]. In adipocytes, the multiple phosphorylations of SIK2 and SIK3 that are induced upon activation of cAMP/PKA-signalling also associate with 14-3-3 proteins. In SIK2, Ser358 is the major site governing the PKA-induced phosphorylation, 14-3-3 binding and intracellular re-localisation from a particulate fraction to the cytosol. This does not result in a change in the intrinsic catalytic activity of SIK2 but is suggested to restrict its actions on downstream targets [56]. In SIK3, several residues cooperate in 14-3-3 binding with a concomitant decrease in catalytic activity [59].

Molecular targets of SIKs

Through their actions on downstream targets, the SIKs are involved in the regulation of a wide range of biological processes, including metabolism, inflammation and cancer [13, 54, 57, 74-82]. Common substrates for the SIKs include transcriptional regulators within the CREB-regulated transcription coactivator (CRTC) and class IIa

histone deacetylase (HDAC) families [76, 83-88]. The regulation of CRTCs and HDACs by SIK isoforms has been confirmed by independent research groups and in several different cell types. Apart from regulation of gene expression, SIKs have also been described to play a more direct role in the control of signal transduction. Additional suggested and potential substrates include IRS1 [11, 66], PI3K regulatory subunit p85 [81], PDE4D [89] and PP2A [90, 91]. However, so far these observations are in most cases made by individual research groups and in specific cell types, and might not be universal to other model systems.

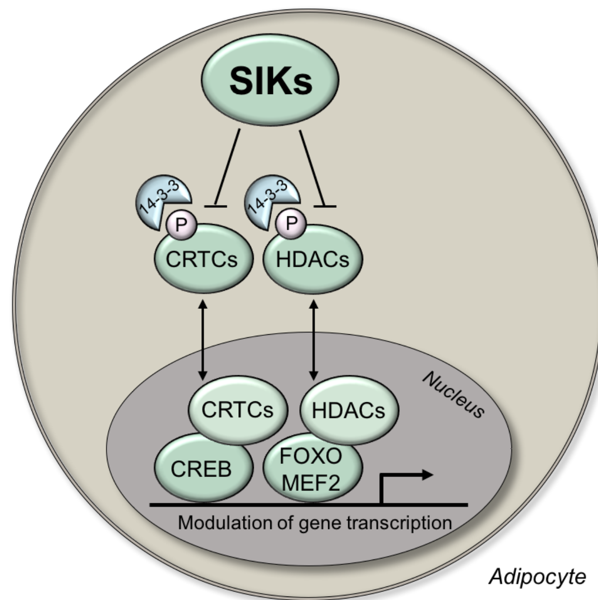

Figure 6. Regulation of CRTCs and HDACs by SIKs.
Tha transcriptional activities of CRTCs and HDACs are controlled by their intracellular localisation. Phosphorylation by SIKs result in binding to 14-3-3 proteins and sequestration in the cytoplasm. Upon dephosphorylation, CRTCs and HDACs translocate to the nucleus and act in transcriptional complexes with CREB and FOXO/MEF2, respectively.

The transcriptional activities of CRTCs and HDACs are controlled by their intracellular localisation. In the basal state, CRTCs and HDACs are sequestered in the cytoplasm by phosphorylation-dependent binding to 14-3-3 proteins. Upon dephosphorylation, 14-3-3 is released and CRTCs and HDACs translocate to the nucleus where they act in transcriptional complexes with CREB [86] and FOXO/myocyte enhancer factor 2 (MEF2) [92], respectively. When present in the nucleus, CRTCs act as transcriptional coactivators [86], whereas HDACs generally act as transcriptional corepressors [92]. Several phosphorylation sites that are regulated by the AMPK-family of kinases have been identified in CRTCs and HDACs (Figure 6).

CREB-regulated transcription coactivators (CRTCs)

The CRTC family consist of CRTC1, CRTC2 and CRTC3 (previously known as transducers of regulated CREB [TORCs]) [86]. The major SIK-dependent phosphorylation site in CRTC2 is Ser171 [65, 84] that acts as a gatekeeper site for the binding of 14-3-3 and consequently the cellular activity of CRTC2 [85]. Ser171, and two additional sites (Ser70 and Ser275) are functionally conserved in all CRTC isoforms and are targeted for phosphorylation by AMPK-RKs [85]. Ser171 and Ser275 in CRTC2 were identified to be phosphorylated by MARK2 and regulate its cellular activity in β-cells and islets in response to glucose [93]. Moreover, Uebi et al (2010) identified an additional site in CRTC2 (Ser307) to be phosphorylated by SIK1 and MARKs *in vitro* [94]. In macrophages, Ser62, Ser162, Ser329 and Ser370 in CRTC3 was identified as SIK-dependent phosphorylation sites involved in the regulation of 14-3-3 binding, intracellular localisation and CREB-dependent transcriptional activation [76]. Ser62 and Ser162 are conserved between CRTC isoforms, whereas Ser329 and Ser370 are unique to CRTC3.

Class IIa histone deacetylases (HDACs)

The class IIa HDAC family consists of HDAC4, HDAC5, HDAC7 and HDAC9. Their sequences contain an N-terminal adapter domain harbouring a MEF2 binding site and conserved phosphorylation motifs serving as 14-3-3 binding sites [92]. Binding of 14-3-3 proteins to class IIa HDACs exposes a nuclear export sequence (NES) while masking a nuclear localisation signal (NLS), promoting their accumulation in the cytoplasm [95, 96]. Kinases of the AMPK family phosphorylate class IIa HDACs on conserved serine residues in their N-terminal domains – Ser246 (HDAC4), Ser259 (HDAC5), Ser155 (HDAC7) and Ser220 (HDAC9) – to regulate their nucleocytoplasmic shuttling and downstream transcriptional activities [87, 88, 97, 98].

Protein phosphatase 2A (PP2A)

SIK2 has also been reported to interact with the catalytic subunit of PP2A and form a complex that functions to preserve both kinase and phosphatase activities [90]. Li et al (2015) identified that SIK2 also bind the regulatory B subunit that resulted in increased SIK2 protein stability [91].

SIK consensus phosphorylation motif

The canonical AMPK-RK consensus phosphorylation motif (L-x-[HKR]-[ST]-x-<u>S</u>-x(3)-L (phosphorylated residue underlined) [84, 99] partly confers to the recognition sequence for binding of 14-3-3 proteins which contain arginine at the -3/-4 position relative to the phosphorylated residue [73, 100]. Consequently, SIK-dependent phosphorylation of a substrate is often associated with 14-3-3-binding [84, 88, 99]. Recently, a variant AMPK-RK phosphorylation motif was suggested (<u>S</u>LPDL), that

lack N-terminal basic residues (arginine). Nonetheless, these motifs are phosphorylated by AMPK-RKs as well as capable of binding 14-3-3 proteins. The Ser70 and Ser275 residue in CRTC2 correspond to this variant motif [85]. This suggest that the range of cellular targets downstream the AMPK-RK is likely more extensive than previously anticipated. Importantly, this might also contribute to the identification of novel SIK substrates.

Roles of SIKs in the regulation of metabolism

From their similarities to AMPK, SIKs are believed to play an important role in the regulation of metabolic processes [13]. Through their ability to control CRTCs, SIKs are involved in the regulation of CREB-dependent gene transcription in tissues that respond to hormonal and nutrient signals [57, 61, 64, 65, 75, 84, 101, 102]. CREB is typically activated upon increasing intracellular cAMP levels, for example in response to catecholamine or other starvation signals, through PKA-mediated phosphorylation at Ser133. CREB act in transcriptional complexes with the CREB-coactivators CRTCs. The phosphorylation status of CRTCs acts as an on/off-switch for CREB-dependent gene transcription by controlling the nucleocytoplasmic shuttling of CRTCs. The cellular activity of SIKs towards CRTCs determines the degree of phosphorylation, and thus CREB-activity on target promoters [86].

Gluconeogenesis

A major target tissue for the actions of CREB is the liver where it stimulates the expression of gluconeogenic genes during fasting. Key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), and the transcription factor peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1-α) are direct CREB-target genes [86]. The involvement of SIKs as negative regulators of hepatic gluconeogenesis via CRTC2 have been demonstrated in several reports [65, 94, 99]. Recent studies have delineated that SIK1 [102] and SIK2 [57, 75] are dispensable for the hormonal control of gluconeogenesis in the liver and that SIK3 instead plays an important role in the regulation of this process [61]. Interestingly, SIKs have also been connected to the regulation of gluconeogenesis through their actions on HDACs [98, 103]. Similar to CRTCs, a reduced cellular activity of SIKs towards class IIa HDACs during fasting conditions resulted in their nuclear translocation. In this setting HDACs acted as transcriptional *activators* by deacetylation of FOXO transcription factors to induce the expression of G6Pase [98].

Glucose uptake

Both CRTCs and HDACs have been linked to the regulation of glucose uptake through their control of GLUT4 expression in muscle [97] and adipocytes [75, 104, 105]. CRTCs and HDACs act, either directly or indirectly, as transcriptional

repressors of *GLUT4* expression. When present in the nucleus, HDACs repress the MEF2-induced transcription of *GLUT4* [92]. AMPK has been suggested as an upstream kinase of HDAC5 in muscle [97] but in adipocytes the mechanism underlying the nucleocytoplasmic shuttling of HDAC4/5 was not elucidated [105]. Thus, the role of SIKs in regulating GLUT4 expression via HDACs remains to be investigated. On the other hand, increased activity of CREB in obese mice has been reported to inhibit *Glut4* expression in adipocytes via an indirect mechanism that involves the stimulation of activating transcription factor 3 (ATF3) expression. ATF3 in turn suppresses the expression of adiponectin and GLUT4 to promote systemic insulin resistance [104]. A similar *Atf3*/*Glut4* phenotype was mimicked by global deletion of SIK2 in mice and deletion of CRTC2 reversed these effects suggesting that the SIK2/CRTC2/CREB-pathway is an important regulator of whole-body energy homeostasis [75].

Lipid metabolism

In addition to their role in the regulation of glucose homeostasis, SIKs have also been described to control lipid metabolism [67, 74, 75, 103, 106]. In adipocytes, SIK2 was observed to inhibit lipogenic gene expression, possibly by promoting the accumulation of sterol regulatory element-binding protein 1 (SREBP-1) in the cytoplasm [12]. Correspondingly, SIK1 inhibits the expression of lipogenic genes in the liver via a similar mechanism [107]. Bricambert et al (2010) suggested a distinct mechanism whereby SIK2 regulates lipogenesis in the liver, by phosphorylation of the HAT p300 at Ser89 to inhibit carbohydrate-responsive element-binding protein (ChREBP)-mediated lipogenesis [108]. Global SIK2 knockout (KO) mice displayed multiple defects in lipid metabolism in adipose tissue (increased expression of lipogenic genes in liver and WAT, decreased FA uptake and increased lipolysis) that contributed to increased plasma triglyceride levels. Together, this implicates that SIK2 is an important regulator of lipid homeostasis. Another global SIK2 KO model was previously described, but a metabolic phenotype has not been reported in these mice [69, 109, 110]. Global KO models have also been reported for SIK1 and SIK3. Recently, two separate global SIK1 KO mouse models were described. Common to both SIK1 KO lines, the mice display lower BW, improved glucose tolerance on HFD and increased plasma insulin [89, 102]. Moreover, muscle-specific KO of SIK1 improved insulin sensitivity on HFD [102]. Global deletion of SIK3 causes a malnourished phenotype due to the reduced expression of genes involved in FA synthesis in the liver [74]. Studies in *Drosophila* have demonstrated that the fly homolog of SIK3 (dSIK3) is a regulator of lipid homeostasis through an HDAC4/FOXO-pathway and the downstream target gene, the *Drosophila* homolog of ATGL (*brummer*, *bmm*) [67, 103]. In brown adipocytes, SIK2 was suggested to inhibit thermogenic gene expression (*Ucp1*) [64].

Insulin secretion

Interestingly, a role has also been suggested for SIKs in the regulation of insulin secretion in pancreatic β-cells [60, 89, 111]. Using an inducible, β-cell-specific SIK2 KO model, Sakamaki et al (2014) demonstrated that SIK2 was required for glucose-stimulated insulin secretion and functional compensation in animal models of T2D [111]. In contrast, another study identified that SIK1 act as a negative regulator of insulin secretion by phosphorylation and activation of PDE4D in β-cells, which subsequently terminated cAMP/PKA-signalling [89]. This was suggested to be an important inhibitory feedback mechanism to reduce insulin secretion in the basal state. Furthermore, SIKs were reported to control insulin secretion through a mechanism involving CRTC1-dependent regulation of miR-212/miR-132 expression [60].

Regulation of the insulin signalling pathway

SIK2 has been connected to the regulation of the insulin signalling pathway itself at different levels. In the first report describing mammalian SIK2, Horike et al (2003) suggested that insulin receptor substrate 1 (IRS1) was phosphorylated by SIK2 at Ser789 (equivalent to human Ser794) *in vitro* and implied that SIK2 might be involved in the inhibition of insulin sensitivity in adipose tissue [11]. Ser/Thr phosphorylation of IRS is believed to modulate the efficiency of the insulin signalling pathway, both positively and negatively [22]. However, due to the complexity of the IRS phosphorylation pattern a consensus has not been reached with regards to the specific roles of individual phosphorylation sites. A link between SIK2 and IRS1 was also discovered in retinal glial cells where SIK2 interacted with and phosphorylated IRS1 in an insulin-dependent manner, but the specific phosphorylation site(s) was not investigated [66]. In these cells, SIK2 was identified as a negative regulator of PKB/Akt phosphorylation [66]. On the other hand, Ahmed et al (2010) demonstrated that the expression of SIK2 was required to promote PKB/Akt phosphorylation in ovarian cancer cells [112]. Recently, the regulatory p85 subunit of PI3K was identified as a substrate of SIK2 *in vitro* and in ovarian cancer cells. SIK2 phosphorylated p85 at Ser154, which subsequently increased the activity of the PI3K/PKB/Akt-pathway [81]. The insulin signalling pathway was also reported to be perturbed upon disruption of SIK2 expression in mice where chronic deletion or overexpression of SIK2 in adipocytes demonstrated that SIK2 promoted the phosphorylation of PKB/Akt and its downstream target AS160 [75].

Roles of SIKs in the regulation of other biological processes

Inflammation

In recent years, SIKs have gained increased attention for their role in the regulation of inflammation. Clark et al (2012) and MacKenzie et al (2013) identified that SIKs, in particular SIK2, promote a pro-inflammatory macrophage phenotype via phosphorylation of CRTC3. Activation of cAMP/PKA-signalling, and genetic or pharmacological inhibition of SIKs, inhibited the cellular activity of SIK2 on CRTC3 and resulted in a CREB-dependent transcriptional switch promoting the presence of anti-inflammatory, regulatory macrophages [58, 76]. A recent study demonstrated that SIK inhibition during macrophage differentiation promoted a stable reprogramming to an anti-inflammatory phenotype even after removal of the treatment [54]. Another study, using SIK-deficient mice, reported that SIK3 was the primary isoform contributing to the anti-inflammatory response *in vivo* [113]. To date, several pharmacological inhibitors of SIKs have been developed and are in trial for the treatment of inflammatory and autoimmune diseases [77, 114].

Cancer

The upstream SIK activating kinase LKB1 is classified as a tumour suppressor and genetic mutations cause Peutz-Jeghers syndrome, characterised by gastrointestinal polyps and mucocutaneous pigmentation, and a high risk of developing gastrointestinal cancer [115, 116]. This has led to the investigation of the potential role of SIKs in cancer. Altered expression levels of SIKs have been reported in various cancer forms, but the downstream effectors often remain to be elucidated. Downregulation of SIK1 is associated with increased growth and metastatic potential in breast cancer [78], ovarian cancer [117] and hepatocellular carcinoma (HCC) [118]. The downregulation in ovarian cancer was suggested to be mediated by miR-141 [117] and the underlying mechanism associated with increased pathogenicity in breast cancer was suggested to involve SIK1 as a regulator of p53-dependent anoikis [78]. On the other hand, increased expression of SIK2 and SIK3 is associated with poor survival of ovarian cancer [79, 81, 112, 119], breast cancer [62, 80] and colorectal tumours [120]. The upregulation of SIK2 promote chemoresistance towards anti-cancer drugs [119, 120] and inhibit autophagic flux [80]. The mechanism underlying the increased SIK2 expression, at least in colorectal tumours, was suggested to be mediated by downregulation of miR-203 [120]. SIK3 promotes cell proliferation, inflammation and metastasis [62, 79, 121]. Development of isoform-selective inhibitors of SIK2 and SIK3, and activators of SIK1, might prove useful for future cancer therapies.

Other biological roles of SIKs

In addition to the biological functions described above, SIK1 has been demonstrated regulate steroidogenesis in adrenal glands [52, 122], intracellular sodium sensing and transport [70, 123-126] and muscle differentiation [68, 87]. SIK2 is a negative regulator of neuronal survival [69] and suppresses melanogenesis [109]. Moreover, SIK2 has been reported to promote autophagy [127, 128] and stimulate ER-associated protein degradation (ERAD) through the interaction with and phosphorylation of p97/valosin-containing protein (VCP) [129]. SIK3 is important for skeletal development [130, 131], while SIK2 suppress bone formation [132].

AIMS

The overall aim of my project is to study the expression, regulation and biological functions of salt-inducible kinases (SIKs), in particular SIK2, in (human) adipose tissue. The specific aims were to:

- Identify cAMP/PKA-regulated molecular targets of SIK2 in adipocytes
- Investigate the expression of SIKs in human obesity and insulin resistance
- Investigate the regulation of SIK2 in response to insulin in adipocytes
- Investigate the role of SIKs in the regulation of insulin signalling and glucose uptake in adipocytes

METHODOLOGY

Adipocyte cell models/Adipose tissue

The work in this thesis was performed using several adipocyte cell models, both primary and cultured cells of rodent and human origin. A reason for employing different models is their compatibility with different types of experimental procedures, further discussed below. However, confirming a research question in cell models of different origins is also an important way of validating and strengthening the results. When possible, we have used human model systems because of their higher physiological relevance, and because comprehensive studies of SIKs in human adipocytes have not been performed previously.

Primary, mature adipocytes

Primary, mature adipocytes were prepared from adipose tissue biopsies by collagenase digestion, and subsequently separated from the stromal-vascular fraction (SVF). The SVF contains non-adipose cells such as progenitors, supportive cells and immune cells. A defining characteristic of primary adipocytes is that they contain a large intracellular lipid droplet that constitutes the majority of the cell volume (Figure 7a). This gives primary adipocytes special properties in form of buoyancy that allows them to float on top of culture media [133]. The advantages of using freshly isolated primary adipocytes are that they more closely resemble the *in vivo* situation and they are better suited for adenoviral transduction compared to commonly used adipocyte cell lines. However, primary adipocytes cannot be kept in culture for extended periods of time due to their inability to attach to surfaces and the potential to use silencing strategies, that require longer incubation times for protein turnover to occur, is thus limited in these cells.

Rodent adipocytes

Primary rodent adipocytes were isolated from epididymal fat pads of male Sprague-Dawley rats or C57BL/6J mice. The epididymal fat pad is a vWAT depot that often is used because of its accessibility and abundancy compared to other depots [134]. However, there is no directly corresponding adipose tissue depot in humans and

given the structural and functional differences between adipose tissue depots this raises the concern of the translatability of the results [2, 134]. Relatively young adult animals (6-week-old rats, 12-14-week-old mice) were used in order to retain insulin sensitivity of the cells. Male rodents were preferably used to eliminate potential influences of female reproductive hormones on adipose tissue physiology [134, 135].

Human adipocytes

Primary human adipocytes were isolated from abdominal subcutaneous or visceral (omental) adipose tissue from patients undergoing different surgical procedures (bariatric surgery, cholecystectomy or reconstructive breast surgery [DIEP Flap]). Adipose tissue biopsies were obtained from Skåne University Hospital (in collaboration with Eva Degerman/Mikael Ekelund and Karin Lindkvist). A major advantage with using primary human adipocytes is that it is the model system that most closely mimics the *in vivo* situation. Potential drawbacks with using primary human adipocytes are the larger interindividual variation, due to biological factors, compared to primary rodent adipocytes or cultured adipocytes. Moreover, the environmental conditions and time delay during the transportation of biopsies from hospital to laboratory likely also impact the quality of the adipocytes between different preparations. Since the biopsies were only used to obtain primary adipocytes for acute experiments, the subjects were not selected based on any criteria. This means that they are not matched with regards to age, gender or other clinical parameters. From a positive side, a finding in unmatched samples likely points to a more robust and physiological effect. One consequence of variation is that a larger number of individuals are generally required to make accurate comparisons. Another disadvantage with using human material is the irregular and sometimes limited supply of tissue specimens.

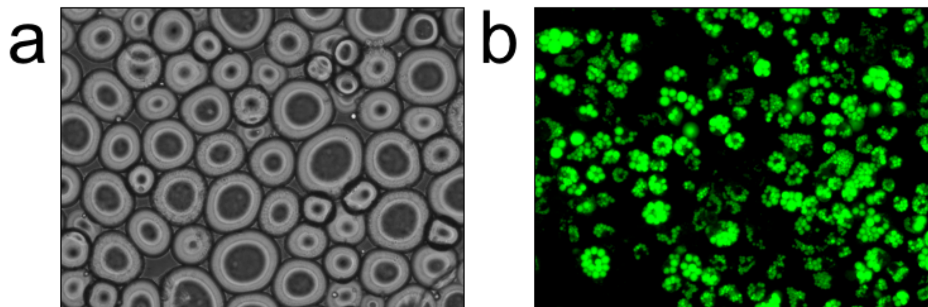

Figure 7. Microscope images of adipocytes.
(**a**) Primary, human adipocytes isolated from scWAT and visualised under the light microscope. Each cell displays a single, large intracellular lipid droplet. (**b**) Fully differentiated 3T3-L1 adipocytes stained with the lipid binding dye BODIPY and visualised under the fluoresence microscope. Each cell displays multiple lipid droplets, that is characteristic of cultured adipocytes differentiated *in vitro*.

In vitro differentiated adipocytes

Cultured adipocytes differentiated *in vitro* were used for their practicality in large-scale experiments or when the choice of experimental method made primary cells less appropriate to use. Advantages are that they can be cultured for longer time periods compared to primary cells offering the potential to use silencing strategies, and that the biological variation is generally low, compared to primary cells, due to the standardised culturing conditions. To employ cultured cells is also a way of reducing the number of experimental animals used for research. Morphologically, adipocytes differentiated *in vitro* display a characteristic appearance with multiple intracellular lipid droplets (Figure 7b). Drawbacks with using cultured cells are that they are not present in a physiological environmental context which might be a cause of their distinct adipogenesis and morphology [136]. In turn, this might influence their functional properties.

3T3-L1 adipocytes

In papers I and II, we used 3T3-L1 adipocytes to perform siRNA silencing and mechanistic studies. The 3T3-L1 is a murine cell line of fibroblast-like preadipocytes that have the capacity to terminally differentiate into mature adipocytes after induction with a differentiation cocktail. The differentiation cocktail is a mixture of the glucocorticoid dexamethasone, insulin and the cAMP-elevating agent isobutylmethylxanthine (IBMX) that is known to induce adipogenesis [133, 137]. Differentiation is accompanied by an increased expression and activity of enzymes involved in *de novo* FA and TAG synthesis resulting in an increased lipid accumulation [138-140]. The 3T3-L1 cell line is well characterised and the most widely used model system to study the molecular events involved in differentiation and physiology of adipocytes [133, 137]. A drawback is that 3T3-L1 cells are of murine origin.

Human mesenchymal stem cell derived adipocytes

To be able to perform silencing of SIKs in human adipocytes we used human adipocytes differentiated *in vitro* from mesenchymal stem cells isolated from the adipose SVF. Morphologically they resemble 3T3-L1 adipocytes with multilocular intracellular lipid droplets, but functionally the properties of a human adipocyte are preserved [141, 142]. Experiments on these cells were performed at Karolinska Institutet (Stockholm) as part of a collaboration with Jurga Laurencikiene.

Modulation of SIK expression and activity

To study the role of SIKs in adipocytes we have employed different strategies to modulate SIK expression and/or activity – adenoviral transduction, genetic modulation and pharmacological inhibition.

Adenoviral transduction

In order to study the role of individual phosphorylation sites in the regulation of SIK2 function we used adenoviral vectors to overexpress hemagglutinin (HA)-tagged wild type and site-specific mutants in primary rat adipocytes (paper I). This method requires cell surface expression of the coxsackievirus and adenovirus receptor (CAR) for the cells to be efficiently transduced. 3T3-L1 adipocytes lack CAR which is why primary adipocytes were used for these experiments. An advantage with expressing tagged proteins is the possibility to selectively immunoisolate the exogenous protein. An important consideration, especially when studying the role of individual phosphorylation sites on protein function, is that you need to achieve an appropriate amount of overexpression to mask effects of the endogenous protein, while still preserving a functional regulation of the protein. Disadvantages are that work with viruses requires the use of designated cell culture rooms and equipment, and that the virus infection *per se* might influence adipocyte function. To rule out that the virus infection is interfering with the experiment proper controls should be used, for example viruses encoding an empty vector.

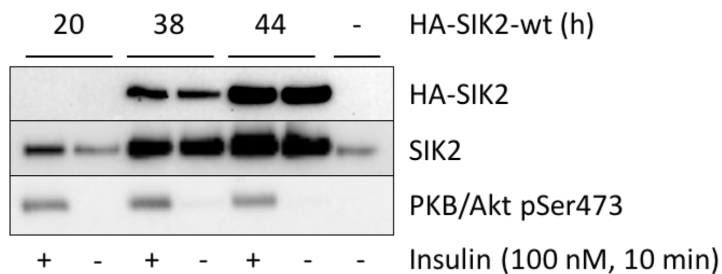

Figure 8. Overexpression of SIK2 in human adipocytes.
Primary human adipocytes were transduced with adenoviruses encoding HA-tagged, wild type SIK2 (HA-SIK2-wt) for different times, followed by stimulation with insulin, as indicated. The expression of SIK2 was determined by western blotting. In the upper panel an antibody towards the HA-tag was used that only recognises the expression of exogenous SIK2 (HA-SIK2). The middle panel displays both endogenous and exogenous SIK2 (SIK2). Phosphorylation of PKB/Akt at Ser473 is shown as a positive control for insulin stimulation.

Recently, we also successfully established this method in primary human adipocytes (Figure 8). Importantly, human adipocytes display a preserved insulin response even after two days in culture (shown as phosphorylation of PKB/Akt at Ser473) compared to rat adipocytes, which gradually lose their insulin sensitivity. This provides the opportunity to employ overexpression strategies also in human cells in the future.

Genetic silencing

siRNA silencing

In papers I and II we performed siRNA silencing of SIKs in 3T3-L1 and *in vitro* differentiated human adipocytes, respectively. Adipocytes are generally difficult to transfect with genetic material due to their unique morphology. For this purpose we used electroporation to achieve silencing at an appropriate efficiency. An advantage with using siRNA silencing is that it enables the study of the role of an individual protein in a specific cell type. However, it is important to optimise the type and concentration of siRNA in order to reduce off-target effects, and incubation times to ensure turnover of the protein of interest. Another problem with the chronic deletion of a protein is the risk of compensatory effects. In human adipocytes differentiated *in vitro* we indeed observed upregulation of both SIK1 and SIK3 when SIK2 was silenced and this resulted in significant amounts of residual SIK activity that made it impossible to draw any conclusions about the specific role of SIK2 in these cells.

SIK2 knockout adipocytes

In paper IV, we also used global SIK2 KO mice that were bred and housed at the University of Copenhagen, Denmark (courtesy of Bente Kiens and Erik Richter). *Sik2*$^{+/lox}$ mice (previously generated [57]) were crossed with EIIa-CRE transgenic mice [143] to produce *Sik2*$^{+/-}$ mice that were subsequently crossed to generate *Sik2*$^{+/+}$ (wild type) and *Sik2*$^{-/-}$ (SIK2 KO). Mice were bred on a C57BL/6J background, the most widely used mouse strain in medical research. *Sik2*$^{-/-}$ have a deletion of exon 5, spanning amino acids 160-201, and containing part of the catalytic domain including the activating Thr175 residue. Experiments with SIK2 KO animals were performed on both males and females. The mice were used at 12-24 weeks of age and in the experiments cells were matched between genotypes with regard to age and gender. Knockout mouse models offer the advantage to study physiological effects of an individual protein *in vivo* compared to using silencing strategies in cells. However, germline deletion of a protein is associated with risk of compensatory upregulation and functional redundancy of other proteins that might mask true effects of the deficiency or give rise to unrelated artefacts. Another disadvantage is the species differences between mouse and human, and thus the translatability of the results.

Pharmacological inhibition

The recent development of pharmacological SIK inhibitors made it possible to study effects of acute kinase inhibition in mature cells without loss of protein expression and associated compensatory mechanisms. Pharmacological inhibition of SIKs is a strategy used in papers I, II and IV, with a focus on the highly selective pan-SIK inhibitor HG-9-91-01 (SIKs: IC_{50} <10 nM) [76]. The specificity of HG-9-91-01 towards SIKs has been elegantly demonstrated by expressing a drug-resistant mutant SIK2 (T96Q) where a threonine residue at the gatekeeper site is replaced by a bulky amino acid (glutamine) [57, 76]. In paper I we also employed a related but less potent inhibitor, MRT199665, that in addition to inhibiting SIKs also acts on AMPK and other AMPK-RKs (MARKs, MELK, NUAK1) [76]. Important considerations when using pharmacological agents are that the inhibitor ideally should be specific towards the kinase studied, and that the dose used needs to be evaluated and optimised for different types of experiments so that off-target effects are minimised. A drawback is that these inhibitors act on all three SIK isoforms and it is therefore not possible to distinguish the relative contribution of the different isoforms.

Western blotting/Phosphorylation site analysis

Western blotting was used in order to evaluate protein expression and site-specific phosphorylation in all papers. Western blotting is commonly used in signal transduction research in order to detect changes in post-translational modifications (phosphorylations). We made use of both commercial and several custom-made antibodies in our analyses. In order to achieve accurate results, it is important to evaluate all antibodies for their specificity, for example by using material where the protein is silenced or cells expressing site-specific mutants. A drawback with using western blotting it is that is only semi-quantitative and it is therefore important to include appropriate experimental controls as well as loading control(s) with all gel runs in order to be able to normalise and compare values from replicated experiments. Moreover, it is not as sensitive in detecting small differences between experimental groups. For the expression study of SIK2 in primary human adipocytes from individuals with varying BMI (paper II) we used both a loading control and an internal control sample that was loaded in the outer lanes of each gel to normalise the values. In addition, the samples were run on gels twice, loaded in different orders, to control for variations in transfer efficiency.

In vitro kinase activity assay

The catalytic activity of a kinase can be altered by phosphorylations in response to different stimuli and is often used as a readout for kinase function. After immunoisolation from a lysate, the kinase is incubated in the presence of a substrate peptide and radioactively labelled ATP. The amount of radioactivity incorporated in the substrate is then recorded in a beta counter. For an accurate analysis, the amount of lysate and immunoprecipitating antibody used in the assay need to be evaluated in order to ensure specificity and linearity. Moreover, the substrate peptide needs to be validated for the use with each specific kinase. The kinase assay is quantitative and thus yields a number of the amount of ATP incorporated in the peptide per unit of time.

Interaction studies

One characteristic of a *bona fide* substrate is its ability to interact physically with the upstream kinase. In paper I, we performed co-immunoprecipitation assays as a step in the characterisation of SIK2 substrates in adipocytes. HA-tagged SIK2 was expressed in primary rat adipocytes followed by immunoisolation and detection of co-immunoprecipitating proteins by western blotting. To ensure specificity in immunoprecipitation assays it is important to use appropriate controls. The purpose of the control is to confirm that the immunoreactive bands on the western blot corresponds to the protein of interest and not an unspecific protein that is pulled down in the control as well. In this case where we analysed exogenously expressed SIK2, cells transduced with adenoviral vectors expressing GFP were used as controls. For endogenous proteins, an unspecific immunoglobulin G (IgG) antibody is generally used. In paper I, we also performed a more unbiased analysis of SIK2-interacting proteins by large-scale purification of SIK2 followed by mass spectrometry.

Glucose uptake assays

In this thesis we evaluated the role of SIKs in the regulation of glucose uptake in adipocytes. Glucose taken up by adipocytes can either be used to synthesise FAs (*de novo* FA synthesis) or glycerol followed by their esterification to form TAGs. The majority of the metabolised glucose is incorporated in the glycerol moiety of TAGs. There are several methods to study glucose uptake and in total three slightly different

protocols have been used. The differences between the protocols are in the type of glucose isotope and scintillation liquid used. First, by using either metabolisable (^{14}C-glucose and ^{3}H-glucose) or non-metabolisable (2-deoxy-^{3}H-glucose) glucose, the fate of the radioactively labelled glucose can be determined. The glucose that is transported inside the cells and not metabolised is present in the aqueous phase, and the glucose that is taken up and incorporated into TAGs and FAs is present in the lipid phase. Second, the choice of scintillation fluid determines in which phase (aqueous or lipid) radioactivity is detected.

In papers I and IV, we measured the uptake of ^{14}C-glucose (30 min) in primary rodent and human adipocytes. Adipocytes were separated from the medium by centrifugation. Cells treated with cytochalasin B, a competitive inhibitor of glucose transporters, was used to assess the amount of glucose present in the adipocyte fraction, but that had not been taken up by the cells. The radioactivity present in the aqueous phase was recorded.

In paper II, we measured the uptake of 2-deoxy-^{3}H-glucose (15 min) or ^{3}H-glucose (2 h) in human adipocytes differentiated *in vitro*. Cells were carefully washed to remove excess glucose that had not been taken up by the cells. The radioactivity present in the aqueous phase was recorded for cells incubated with non-metabolisable 2-deoxy-^{3}H-glucose and in the lipid phase for cells incubated with metabolisable ^{3}H-glucose. The assay using ^{3}H-glucose is based on a lipogenesis protocol. In human adipocytes, glucose uptake is the rate-limiting step for lipogenesis at micromolar concentrations of glucose [144], which means that measuring the radioactively labelled glucose present in TAGs and FAs reflects glucose uptake. The reason for using a longer incubation time is to allow the glucose to be incorporated into lipids.

Gene expression analysis

Real-time reverse transcription polymerase chain reaction (qRT-PCR)

Analysis of gene expression was performed using real-time reverse transcription polymerase chain reaction (qRT-PCR). The procedure is performed in three steps – RNA extraction from cells, reverse transcription of total RNA to cDNA and PCR amplification of cDNA in a PCR reaction. The PCR reaction makes use of gene-specific primers that amplifies the gene of interest and generates a fluorescent signal that is proportional to the amount of transcript. The signal is recorded and then used to calculate relative gene expression using the $2^{-\Delta\Delta Ct}$ method [145]. In addition to the gene of interest, a reference (or housekeeping) gene is included in the reaction. Values are normalised both to the reference gene and to a calibrator (experimental control)

sample. For an accurate analysis, the choice of reference gene(s) need to be evaluated in each model system and/or experimental setting. Ideally, a reference gene should not display any variation between different experimental groups.

Two different techniques were used for detection of the amplification reaction – TaqMan and SYBR Green assays. The TaqMan assay consists of probes that are labelled with a fluorescent dye and a quencher. During amplification the probe is degraded and when the dye is released from its quencher it emits a fluorescent signal, proportional to the number of amplified transcripts. The dye in the SYBR Green assay emits a fluorescent signal upon binding to double-stranded DNA. The SYBR Green dye binds non-specifically to double-stranded DNA which means that it also recognises primer-dimers and unspecific PCR products. Advantages with using TaqMan assays are their higher specificity but they are also more expensive compared to SYBR Green assays.

Absolute quantification of mRNA

In paper II, we performed a detailed analysis of the absolute mRNA expression levels of SIK isoforms in different adipocyte cell models. Standard qRT-PCR is quantitative in the sense that you can compare the expression of a single transcript between different tissues/treatments. However, it cannot be used to directly compare the expression of several different genes due to differences in primer efficiency. In order to quantify the absolute levels of SIK mRNA transcripts in different adipocyte models, we used custom oligonucleotide sequences that were designed to cover the sequence recognised by the PCR primer (≈20 bases) flanked by the surrounding DNA sequence (total ≈100 bases). Standard curves were generated by serial dilution of the oligonucleotides, and then amplified in a standard qRT-PCR reaction, which also included cDNA for the samples. Ct-values were used to create separate standard curves for each target gene and the amount of RNA in the samples was then be calculated from the linear equation.

MAIN FINDINGS

Paper I

- The transcriptional regulators CRTC2, CRTC3 and HDAC4 are direct substrates of SIK2 in adipocytes
- cAMP/PKA-induced phosphorylation of SIK2 at Ser358 is functionally important for controlling the interaction between SIK2 and CRTCs
- SIK2 interacts with PP2A in adipocytes
- SIK2-signalling regulates GLUT4 expression and glucose uptake in adipocytes

Paper II

- *SIK2* and *SIK3* mRNA are downregulated in adipose tissue from obese individuals, and the expression is increased by weight loss
- *SIK2* and *SIK3* mRNA are negatively correlated with systemic insulin resistance, independently of BMI and age for *SIK2*
- SIK2 protein levels and catalytic activity display a negative association with BMI in human adipocytes
- The expression of SIK2 and SIK3 is downregulated by inflammation (TNF-α) in adipocytes
- SIKs are involved in the regulation of glucose uptake in human adipocytes, likely via promoting PKB/Akt phosphorylation and GLUT4 localisation to the plasma membrane

Paper III

- SIK2 is phosphorylated at a specific residue (Thr484) in response to insulin in adipocytes
- Phosphorylation at Thr484 is not associated with a change in the catalytic activity of SIK2
- Insulin stimulation stabilises SIK2 protein levels in adipocytes, likely via reducing proteasomal degradation

Paper IV

- SIKs promote glucose uptake in primary human adipocytes
- The underlying mechanism involves positive, and direct, effects on the insulin signalling pathway, likely downstream of IRS1
- SIK2 is important for maintaining normal glucose uptake in mouse adipocytes

RESULTS AND DISCUSSION

Characterisation of cAMP/PKA-regulated molecular targets of SIK2 in adipocytes (paper I)

An important step in determining the biological functions of SIK2 in adipose tissue involves the identification of cellular substrates. In other tissues, SIKs typically regulate gene expression through controlling the phosphorylation of transcriptional regulators [76, 83-88]. Identification of direct substrates of SIK2 in adipocytes also serves the purpose to establish a readout for the cellular activity of SIK2, since the intrinsic kinase activity is not altered in response to hormonal stimulation in these cells [56]. In paper I we investigated whether the CREB co-activators CRTC2 and CRTC3, and the class IIa histone deacetylase HDAC4 are substrates of SIK2 in adipocytes, and if the cAMP-dependent regulation of CRTCs and HDAC4 is mediated via the PKA-phosphorylation of SIK2 at Ser358. A *bona fide* kinase substrate needs to fulfil several criteria, including changes in its phosphorylation when modulating expression/activity of the suggested upstream kinase and a direct interaction between kinase and substrate [146].

CRTCs and HDAC4 are phosphorylated by SIK2 in adipocytes

Silencing of SIK2 or pharmacological inhibition of SIKs in adipocytes results in a *decreased* total and site-specific phosphorylation of CRTC2 (Ser275), CRTC3 (Ser162) and HDAC4 (Ser246) as determined by changes in electrophoretic mobility and by using phosphospecific antibodies. On the other hand, adenoviral overexpression of wild type SIK2 *increases* the phosphorylation of these proteins. Moreover, we demonstrate that the stimulation of these phosphorylations are dependent on SIK2 kinase activity since expression of a kinase inactive mutant of SIK2 (Thr175Ala) fails to induce substrate phosphorylation. Residual phosphorylation of CRTCs and HDAC4 is still present after SIK2 silencing or SIK inhibition indicating that additional kinases also contribute to the basal phosphorylation of these proteins. In addition, our analysis was directed towards specific sites, known to be controlled by SIKs, and it is possible that other unknown sites are phosphorylated by SIK2. We attempted to identify SIK2-dependent

phosphorylation sites in CRTC2 using mass spectrometry. Unfortunately, we were unsuccessful due to technical issues.

CRTCs, HDAC4 and PP2A interact with SIK2 in adipocytes

In paper I, we demonstrate that CRTC2, CRTC3 and HDAC4 all co-immunoprecipitate with SIK2, further strengthening the hypothesis that these are direct molecular targets of SIK2 in adipocytes. We also employed mass fingerprinting, a more general and unbiased approach to identify additional interacting proteins of SIK2. In this screen, we found it particularly interesting that subunits of the protein phosphatase 2A (PP2A) bind SIK2. We demonstrate that both regulatory A and B, and the catalytic C subunit of PP2A interact with SIK2 in adipocytes. PP2A has been reported to interact with SIK2 in HEK293T cells and this complex formation was important to preserve both kinase and phosphatase activities [90]. Later, SIK2 was also found to interact with PP2A B subunit in glioma cells leading to increased SIK2 protein stability and PP2A phosphatase activity [91]. The exact role of the SIK2-PP2A complex in adipocytes remain to be elucidated.

The cellular activity of SIK2 towards downstream substrates is regulated by cAMP/PKA-signalling

The cellular function of SIK2, CRTCs and HDACs is regulated by phosphorylation-dependent binding to 14-3-3 proteins. The phosphorylations at Ser275 (CRTC2), Ser162 (CRTC3) and Ser246 (HDAC4) are all known to bind 14-3-3 proteins [76, 85, 88, 93]. In paper I we observed that the phosphorylation at these sites were reduced in response to activation of cAMP/PKA-signalling in adipocytes. Phosphorylation is reduced to similar levels after cAMP-induction as after SIK2 silencing indicating that SIK2 is the major kinase responsible for maintaining the phosphorylation of these proteins in the basal state. However, phosphorylation is not completely ablated following either silencing of SIK2, SIK inhibition or cAMP-induction, indicating that additional kinases likely contribute to the phosphorylation of these proteins. Stimulation with cAMP-elevating agents typically reduce the phosphorylation of CRTC2 and HDAC4 to a greater extent compared to silencing of SIK2 alone, suggesting that other CRTC and HDAC kinases also display cAMP/PKA-dependent regulation. AMPK, and other AMPK-RKs, have also been reported to phosphorylate CRTCs and HDACs [45, 57, 93, 97] and it is possible that these kinases also contribute to CRTC and HDAC phosphorylation in adipocytes. Moreover, activation of cAMP/PKA-signalling was associated with a reduced interaction of SIK2 to CRTC2, CRTC3 and PP2A.

The PKA-induced phosphorylation of SIK2 at Ser358 is responsible for mediating binding to 14-3-3 proteins and subsequent intracellular relocalisation [56] but it is not known how this phosphorylation influence the cellular activity of SIK2 in adipocytes. In paper I we addressed the functional importance of SIK2 Ser358 phosphorylation for the cAMP-dependent regulation of CRTCs and HDAC4 by expressing a cAMP-resistant Ser358Ala mutant. Surprisingly, the cAMP/PKA-dependent decrease in phosphorylation of CRTC2 and HDAC4 is unaffected by mutating Ser358. Unfortunately, we were not able to confidently detect site-specific phosphorylation of CRTC3 in the primary cells used for these experiments. However, the reduced interaction of SIK2 to CRTC2 and CRTC3 upon cAMP/PKA-activation is dependent on Ser358 phosphorylation. SIK2 also bind HDAC4 but this interaction is insensitive both to cAMP-elevation and Ser358Ala mutation. Together, this indicates that PKA-phosphorylation of SIK2 at Ser358 is important for mediating certain aspects of SIK2 function towards specific targets. However, since effect on substrate phosphorylation was not affected by Ser358Ala mutation it is possible that other sites contribute to the cAMP-dependent regulation of CRTCs and HDAC4 in adipocytes. Moreover, the degree of expression of SIK2 Ser358Ala mutant might not have been sufficient to mask effects of the endogenous protein on the substrates.

The data presented in paper I, in combination with previous reports on *in vitro* phosphorylation and matching consensus sequence [84, 87, 98], provide strong evidence that CRTCs and HDAC4 are direct substrates downstream SIK2 in adipocytes. In our following work, we therefore commonly used the phosphorylation status of these proteins as a readout for SIK activity in adipocytes.

Expression of SIK isoforms in human obesity and insulin resistance (papers II and IV, and unpublished data)

An analysis comparing expression and activity of the three SIK isoforms in adipocytes has not been performed previously, and would provide useful information about the relative contribution of each isoform in these cells. In turn, this would facilitate the interpretation of experiments using pharmacological pan-SIK inhibitors.

SIK2 is the dominant SIK isoform in human adipocytes

In paper II we determined the absolute mRNA levels and *in vitro* kinase activity of SIK1-3 in two different human adipocyte models. SIK2 was clearly the most abundant isoform on both mRNA expression and activity level supporting that SIK2

is the dominant isoform relative SIK1 and SIK3 in human adipose tissue. Surprisingly, SIK3 contributed to a higher degree to the total SIK activity (measured by *in vitro* kinase assay and phospho-HDAC4 after siRNA silencing) than anticipated from the mRNA expression analysis. An important conclusion from these data is that a functional assay is a better predictor of the relative contribution of the different SIK isoforms to the cellular activity, compared to mRNA expression levels. Discrepancies might exist between mRNA and protein expression depending on differences in mRNA and protein stability or protein translation. Unfortunately, we were not able to do the corresponding analysis for SIK1 due to the lack of immunoprecipitating SIK1 antibodies.

We also performed an mRNA expression analysis in the other model systems that we generally use – cultured 3T3-L1 adipocytes and primary rat adipocytes (Figure 9, unpublished data). The results demonstrate that in 3T3-L1 adipocytes the expression pattern looks different compared to in human cells, with *Sik1* being clearly more abundant relative to *Sik2*.

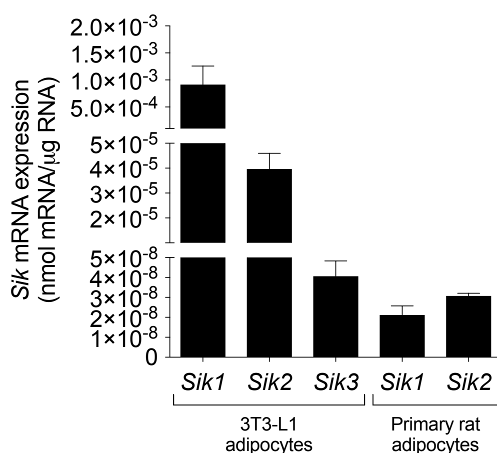

Figure 9. ***Sik*** **mRNA expression in rodent adipocytes.**
Absolute levels of *Sik* mRNA in cultured murine 3T3-L1 adipocytes and isolated primary rat adipocytes, determined by absolute quantification.

In contrast to SIK2 and SIK3, *Sik1* is known to be transcriptionally activated in response to stimuli (most notably cAMP/PKA [87, 89, 147]) and a possible explanation for its high expression in 3T3-L1 adipocytes might be that the composition of the culture media or the culturing conditions activate these signalling pathways and thus the transcription of *Sik1*. Moreover, one should keep in mind that differences between cell models might in part depend on differences in cell sizes and RNA content and it would be more reasonable to compare the number of transcripts normalised per cell or DNA content instead. In primary rat adipocytes, the absolute

expression of *Sik2* was comparable to that in human adipocytes. Unfortunately, we were not able to determine the expression of *Sik3* in primary rat adipocytes due to technical issues.

SIK2 and SIK3 are downregulated in adipose tissue from obese or insulin-resistant humans

The findings by Horike et al (2003) that SIK2 was differentially expressed in WAT of obese, diabetic mice and involved in the modulation of insulin signalling in adipocytes [11] sparked an interest in SIK2 and its potential role in the development of insulin resistance and T2D. However, studies on SIK2 in adipocytes have exclusively been performed in rodents and the expression of SIK2 from a diabetes perspective has not been addressed in humans. In paper II we therefore investigated the expression of SIKs in relation to human obesity and insulin resistance. In contrast to the observations in obese diabetic (*db/db*) mice, we found that the expression of *SIK2*, and also *SIK3*, mRNA was markedly *downregulated* in WAT from obese humans, and that the expression of *SIK2* and *SIK3* was increased in response to weight loss (through bariatric surgery) of the obese individuals. Moreover, *SIK2* and *SIK3* mRNA displayed a negative association with insulin resistance (measured by HOMA-IR). This finding was independent of BMI and age for *SIK2*. For SIK2 we also analysed the protein expression and *in vitro* kinase activity in adipocytes isolated from individuals with varying BMI and in contrast to the findings in mice, but in line with the findings on mRNA expression in human adipose tissue, we found that SIK2 protein and *in vitro* kinase activity both displayed a negative association with BMI. In contrast to our findings, and in line with the findings by Horike et al (2003), SIK2 protein expression was increased in the livers [108] and pancreatic islets [111] of *ob/ob* or HFD-fed compared to wild type mice. Together, this demonstrates that important interspecies differences exist in the regulation of *SIK* expression in the context of obesity and insulin resistance. Similar to SIK2 and SIK3, AMPK is also differentially regulated in the context of human obesity and insulin resistance. AMPK activity is reduced in obese and insulin-resistant humans [148, 149], and concomitantly increases with weight loss [150, 151]. The analysis of *SIK* mRNA expression in human adipose tissue was performed on female subjects only and we at first speculated that there might be gender-specific effects influencing the results. However, the analysis of SIK2 protein in primary human adipocytes was performed with cells from both female and male subjects and the decrease in expression was still preserved in this setting, although with slightly larger variation. In part, this variability likely depends on factors that are inherent to the method of analysis, and indicate that gender might not be a critical factor for the effect on SIK2 expression. Moreover, for the dataset used to analyse SIK2 protein we only had limited access to the subjects' clinical parameters and we therefore cannot exclude that determinants

other than BMI (e.g. insulin sensitivity, inflammation) might be better predictors of SIK2 protein levels in human adipocytes. Contrary to *SIK2* and *SIK3*, we also showed that the expression of *SIK1* mRNA was *upregulated* in WAT from obese humans.

SIK2 expression in human adipose tissue is regulated by inflammation

As adipose tissue inflammation is a common feature of obesity and insulin resistance we hypothesised that *SIK* expression in human adipose tissue might be regulated by inflammatory cytokines. In paper II we investigated whether tumour necrosis factor-α (TNF-α) is involved in mediating changes in SIK expression in adipocytes. We observed that SIK2 mRNA and protein were downregulated in response to treatment with TNF-α in adipocytes. The effect was rapid and SIK2 was significantly reduced on protein level already after 3 hours, suggesting that TNF-α likely acts via direct mechanisms to decrease SIK2 mRNA and protein expression. SIK3 protein was also downregulated by TNF-α in adipocytes but the effect appeared after 16 hours of treatment suggesting that SIK2 is more sensitive to the effects of TNF-α in adipocytes. Given the robust effect of TNF-α on the expression of SIK2, we were at first surprised that we did not observe any association between *SIK2* mRNA expression in human adipose tissue and TNF-α levels in serum (data not shown). However, the inflammation associated with obesity and insulin resistance is often described as low-grade and localised to the adipose tissue [27, 28, 35]. Thus, circulating levels of TNF-α might not necessarily correspond to the levels present in the adipose tissue [152], and there might still be a correlation between the TNF-α levels expressed and/or secreted by adipocytes and macrophages in the adipose tissue *per se*.

SIK1 is transcriptionally activated by the cAMP/PKA/CREB pathway [87, 89, 147], transforming growth factor β (TGF-β) signalling [153, 154] or via a MEK/Erk-dependent pathway [155]. However, the molecular mechanisms underlying transcriptional regulation of SIK2 and SIK3 remains largely unknown. To explore potential mechanisms whereby SIK2 transcription is regulated, we performed a basic bioinformatic analysis of transcription factor binding sites in the SIK2 promoter. In order to do this, we used the Match 1.0 tool, which is based on the TRANSFAC® transcription factor binding-motif database [156], and the GeneCards database that lists transcription factors with binding sites within the promoter based on ChIP-Seq evidence from the Encyclopedia of DNA Elements (ENCODE) project [157]. We identified putative binding sites for the inflammatory mediators NF-κB and activator protein 1 (AP-1), and interferon regulatory factor (IRF3) in the SIK2 promoter. When performing a co-expression analysis to identify genes that correlate with SIK2 we found that IRF3 expression displays a strong negative correlation with SIK2 in human adipose tissue (Figure 10a, unpublished data). IRF3 has recently been

reported to be upregulated in adipocytes from obese or diabetic humans where it plays a role in transcriptional regulation of inflammatory genes [158]. The inverse correlation between SIK2 and IRF3 suggest either that IRF3 is a repressor of *SIK2*, or that IRF3 activates the transcription of a target gene that in turn lowers *Sik2* transcription or mRNA stability.

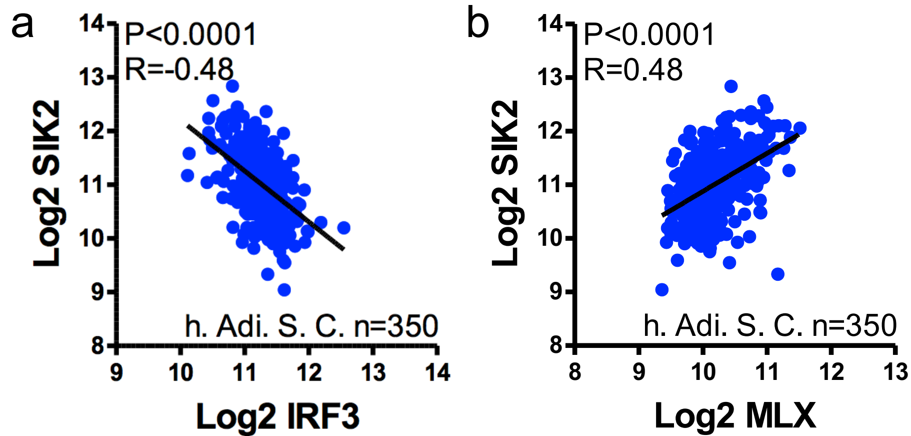

Figure 10. Co-expression analysis in human adipose tissue.
Association between the mRNA expression of *SIK2* and *IRF3* (**a**) or *MLX* (**b**) in human subcutaneous adipose tissue from the GTEx database [55]. Correlations made using Spearman correlation test, R^2=0.23. (*n*=350)

Furthermore, since *SIK2* and *SIK3* mRNA expression was lower in insulin-resistant individuals it is also possible that a functional insulin response is needed for *SIK* transcription. In our transcription factor analysis, we indeed identified putative binding sites for the glucose-activated transcription factor MLX [159] and the insulin-responsive transcription factors SREBP1 [160] and TEAD1 [161] in the SIK2 promoter. Moreover, the expression of MLX displays a strong positive correlation with SIK2 in human adipose tissue (Figure 10b, unpublished data). In line with this hypothesis, the expression of the *Drosophila* SIK3 gene was recently reported to be positively regulated in response to sugar feeding by a transcriptional complex consisting of Mlx and the fly homolog of ChREBP, Mondo, by binding to a carbohydrate response element (ChoRE) in the SIK3 promotor [162]. In human retinal glial cells, chronic hyperglycemia induced *SIK2* mRNA expression [66]. Furthermore, Sakamaki et al (2014) reported that SIK2 is regulated on a post-transcriptional level in response to glucose by increasing SIK2 protein stability in pancreatic β-cells [111].

The chicken or the egg?

An important question to answer is whether differential regulation of SIK expression is a causal factor of obesity and insulin resistance, or the opposite. So far, two different global SIK2 KO mouse models have been described [75, 109]. None of these display any weight phenotype on chow or HFD but the mice described by Park et al (2014) display a disturbed adipose tissue physiology and whole body insulin resistance [75]. These results argue against a protective role for SIK2 in the development of obesity, but implicate that loss of SIK2 might be involved in promoting adipose tissue inflammation and insulin resistance. However, the SIK2 KO mice display adipocyte hypertrophy and an increased adipocyte differentiation capacity and one can speculate that some of the phenotypes might be attributed to changes in the intrinsic properties of the adipocyte itself as a result of the embryonic deletion of SIK2 [75]. Also, some phenotypes might not be penetrant due to functional redundancy among SIK isoforms or other AMPK-related kinases. In paper IV we characterised an independent global SIK2 KO mouse line with regards to metabolic phenotypes. In line with the findings by Park et al (2014), these mice displayed normal body weight regulation and had similar lean- and fat mass compared to wild type mice, and accordingly do not support that downregulation of SIK2 is a causal factor of obesity. Moreover, SIK2 KO mice displayed normal glucose tolerance and plasma insulin levels before and after glucose administration. However, the previously described SIK2 KO model displayed mild glucose and insulin intolerance, even though plasma insulin levels were not changed in response to a glucose challenge [75], making it difficult to draw any conclusions on the exact role of SIK2 in the development of insulin resistance, at least in mice. However, the SIK2 KO mice that we describe were only analysed on a chow diet, and it would be interesting to investigate if there are phenotypes that would penetrate in the context of a HFD. Global SIK3 KO mice have severe metabolic defects and display malnourished phenotypes due to reduced energy storage [74] arguing against a causal role for SIK3 downregulation in the development of obesity and type 2 diabetes. Global deletion of SIK1 renders mice protected from obesity and insulin resistance [89, 102] suggesting that upregulation of *SIK1* might indeed be a causal factor of obesity. However, when silencing SIK2 in human adipocytes differentiated *in vitro* we observed a compensatory upregulation of *SIK1* mRNA (paper II), which means that the increased expression of *SIK1* in obese humans might also be a secondary effect following the downregulation of SIK2.

Role of SIKs in the regulation of insulin signalling and glucose uptake in adipocytes (papers I, II and IV)

The most well characterised function of SIKs is their role in the regulation of gene transcription. The identification of the transcriptional co-regulators CRTC2, CRTC3 and HDAC4 as downstream substrates of SIK2 in adipocytes (paper I), together with their reported involvement in the regulation of *Glut4* expression [104, 105] prompted us to focus on a potential role of SIKs in the regulation of glucose uptake in adipocytes. In summary, we have investigated effects of SIKs on glucose uptake, and upstream mechanisms, in primary or *in vitro* differentiated, human or rodent adipocytes using pharmacological inhibition (papers I, II and IV), adenoviral overexpression (paper I), siRNA silencing (papers I and II) and genetic deletion (SIK2 KO mice) (papers I and IV).

SIKs promote glucose uptake in adipocytes

Due to the role for SIK substrates CRTCs and HDACs as transcriptional regulators we initially focused on effects of more chronic/long-term modulation of SIK expression and activity on glucose uptake. The reason for this was to allow for changes in gene expression to occur downstream SIKs. Overexpression of wild type SIK2 was associated with an increased basal glucose uptake in primary rat adipocytes compared to cells expressing kinase-inactive (Thr175Ala) SIK2 (paper I). Overnight treatment with the highly selective pan-SIK inhibitor HG-9-91-01, reduced both basal and insulin-stimulated glucose uptake in primary rat adipocytes (paper I) and *in vitro* differentiated human adipocytes (paper II). The fold change in glucose uptake induced by insulin was not changed by increasing doses of HG-9-91-01 suggesting that in this setting, SIKs do not affect the relative efficiency of insulin action but rather the absolute capacity to transport glucose, or alternatively that the effects on basal and insulin-stimulated uptake are mediated by separate mechanisms. Furthermore, treatment with a related inhibitor, MRT199665, which inhibits both SIKs and other AMPK-RKs, yielded similar results in primary rat adipocytes (paper I).

Together, the data clearly demonstrated that SIKs are involved in promoting glucose uptake in adipocytes. However, HG-9-91-01 inhibits all SIK isoforms and in order to elucidate the relative contribution of each individual isoform to the positive effect on glucose uptake we performed siRNA silencing in human adipocytes differentiated *in vitro* (paper II). Given the observation that SIK2 is the dominant SIK isoform in human adipocytes, we were at first surprised that silencing of SIK2 did not alter glucose uptake in these cells. However, in this experimental setting we observed

compensatory upregulation of SIK1 and SIK3 expression, which contributed to the presence of residual SIK activity, and made an interpretation of the individual role of SIK2 complicated. Silencing of SIK3 was not associated with compensatory upregulation of the other SIK isoforms and resulted in a reduction of glucose uptake in the basal state demonstrating its importance for maintaining basal glucose uptake in these cells. Similar to SIK inhibition, simultaneous siRNA silencing of all three SIK isoforms resulted in a decreased glucose uptake both in the absence and presence of insulin. Unfortunately, we were not able to achieve a knockdown of SIK1 on protein level in human adipocytes differentiated *in vitro* making it impossible to draw any conclusions about the contribution of SIK1 to the positive effect of SIK isoforms on glucose uptake in these cells.

As an alternative strategy to examine the individual role of SIK2 in the regulation of glucose uptake in adipocytes we used mice with a global deletion of SIK2. Primary adipocytes from SIK2 KO mice displayed decreased glucose uptake compared to wild type both in the basal state and in the presence of insulin (paper IV). The finding that deletion of SIK2 alone was sufficient to lower glucose uptake in mouse adipocytes strengthens the hypothesis that SIK2 indeed plays an important role for the regulation of glucose uptake in adipocytes. In the previously described SIK2 KO model, Park et al (2014) reported that basal and insulin-induced glucose uptake was reduced in muscle, but they did not analyse effects on glucose uptake in primary adipocytes isolated from those mice [75].

Regulation of GLUT4 expression by SIKs

In search for the mechanism whereby SIKs mediated the positive effect on glucose uptake we focused on changes in protein expression that could be regulated downstream SIK2/CRTC2/HDAC4. Silencing of SIK2 using siRNA in 3T3-L1 adipocytes, or global SIK2 KO in mice, resulted in a reduced protein expression of the insulin-responsive GLUT4 in adipocytes (paper I). Silencing of CRTC2 or HDAC4 instead increased GLUT4 expression in 3T3-L1 adipocytes (paper I). In line with our findings, Park et al (2014) reported that *Glut4* mRNA expression was decreased (GLUT4 protein was not analysed) in WAT from SIK2 KO mice compared to wild type, and this was suggested to be mediated by an increased activation of the CRTC2/CREB-pathway [75]. However, acute overexpression or KD of SIK2 in 3T3-L1 CAR adipocytes did not mimic the effects on *Glut4* expression and it is therefore difficult to conclude whether the effects of chronic SIK2 depletion is a result of an alteration in the intrinsic properties of the adipocyte itself rather than reflecting the role of SIK2 in the mature adipocyte in that model [75].

Contrary to our expectations based on the findings in paper I, the expression of GLUT1 and GLUT4 after silencing or pharmacological inhibition of SIKs in human

adipocytes differentiated *in vitro* was not in line with the reduced glucose uptake, and was instead increased (paper II). This made us hypothesise that the positive effect of SIKs on glucose uptake in these cells might instead involve more direct effects on the insulin signalling pathway and/or GLUT4 translocation, rather than changes in gene and protein expression.

SIKs stimulate PKB/Akt phosphorylation in adipocytes

Following overnight SIK inhibition in human adipocytes differentiated *in vitro* we indeed observed that the phosphorylation (and activation) of PKB/Akt at Thr308 and Ser473 was markedly reduced (paper II). The PKB/Akt phosphorylation at Thr308 was also reduced in adipocytes from SIK2 KO mice (paper IV). In line with our findings, the insulin-induced phosphorylation of PKB/Akt was slightly reduced in WAT from the SIK2 KO mice described by Park et al (2014) [75]. However, as discussed above, this effect was not reproduced by acute modulation of SIK2, arguing that some resulting phenotypes of the SIK2 KO mice might be secondary to changes in the function of the adipocyte itself [75]. In contrast to our findings in adipocytes, SIK2 was described as a negative regulator of PKB/Akt phosphorylation in human retinal glial cells [66], suggesting that tissue-specific effects exist.

Having observed that the phosphorylation of PKB/Akt is reduced by overnight SIK inhibition in human adipocytes differentiated *in vitro* we investigated effects of acute (one hour) inhibition of SIKs on glucose uptake, insulin signalling and GLUT4 localisation. Our aim was also to titrate the effect(s) of SIK inhibition on the insulin signalling pathway and glucose uptake in greater detail, by evaluating different doses of insulin. Moreover, to get mechanistic insight we included additional proteins in the insulin signalling pathway in our analysis.

SIKs promote glucose uptake in adipocytes via direct effects on the insulin signalling pathway and GLUT4 translocation

The uptake of glucose in human adipocytes differentiated *in vitro* (paper II), or primary human and rat adipocytes (paper IV) was reduced already after one hour of SIK inhibition suggesting that the positive effect of SIKs on glucose uptake likely involves a direct interaction or modulation of proteins in the insulin signalling pathway. In primary human adipocytes the insulin-induced glucose uptake was completely ablated following one hour of SIK inhibition, indicating a prominent role for SIKs in the regulation of glucose uptake in these cells. Glucose uptake was also reduced in primary rat adipocytes but at a lower potency, which might reflect species differences in the abundancy of SIK isoforms and hence their relative contribution to

the regulation of glucose uptake compared to other pathways. Compared to *in vitro* differentiated human adipocytes (paper II), the effect of acute SIK inhibition in primary cells on glucose uptake was only observed after insulin stimulation, and not in the basal state, likely reflecting model-specific differences in glucose metabolism between primary and cultured cells.

The acute effect on glucose uptake was accompanied by reduced PKB/Akt-phosphorylation in primary human and rat adipocytes (paper IV). The reduction in PKB/Akt phosphorylation was only detected at a submaximal dose of insulin, although a change in glucose uptake was observed for all doses of insulin. This could be explained by methodological differences in the assays used. The analysis of phosphorylation by western blotting is a snapshot at a single time point (10 min of insulin stimulation), whereas the glucose uptake assay measured the cumulative uptake during 30 minutes. Downstream PKB/Akt we consequently observed a reduced phosphorylation of AS160, a key regulator of the GLUT4 translocation machinery, in primary rat adipocytes (paper IV).

Upstream PKB/Akt we analysed serine and tyrosine phosphorylation of IRS1 at several sites but none of these were significantly affected by acute SIK inhibition in primary human or rat adipocytes (paper IV), suggesting that SIKs likely act downstream of IRS1 in these cells, either directly on PKB/Akt or on a signalling component between IRS1 and PKB/Akt in the insulin signalling pathway. Interestingly, a recent report identified that SIK2 phosphorylated the PI3K regulatory subunit p85, which resulted in an increased PKB/Akt phosphorylation in ovarian cancer cells [81]. Another possible mechanism that could account for a reduction in the phosphorylation of PKB/Akt, without apparent changes in IRS1 phosphorylation, is an upregulation of tribbles homolog 3 (TRB3), which directly inhibits the phosphorylation of PKB/Akt [163]. This mechanism was observed in WAT from SIK2 KO mice [75]. Whether differential expression of TRB3 contributes to altered PKB/Akt phosphorylation in response to SIK modulation in our models needs further investigation. IRS1 has been suggested to be a substrate of SIK2 *in vitro* by phosphorylation at Ser789 (equivalent to human Ser794) [11]. Kuser-Abali et al (2013) also connected SIK2 to the regulation of IRS1 by demonstrating that insulin increased SIK2-IRS1 protein interaction and that SIK2 phosphorylated IRS1 *in vitro* [66]. However, using commercially available tools we have not been able to confidently detect a change in the phosphorylation of IRS1 at Ser789/794 in response to modulation of SIK activity in our models. The analysis of IRS1 phosphorylation by Horike et al (2003) was directed towards the Ser789 residue alone and since IRS1 contains multiple residues that are phosphorylated in response to insulin stimulation it is possible that SIK2 is important for controlling the phosphorylation of additional sites, apart from this residue, in IRS1.

The level of glucose uptake is ultimately dependent on the number of transporters present at the plasma membrane. The presence of GLUT4 in the vicinity of the plasma membrane was reduced after acute inhibition of SIKs in primary rat adipocytes (paper II).

Regulation of SIK2 function by insulin in adipocytes (paper III)

The control of adipocyte function is highly dependent on extracellular hormonal signals, the major being the opposing catecholamines and insulin. Catecholamines activate cAMP/PKA-signalling and the regulation of SIK2 in response to this pathway in adipocytes has been well characterised in previous work from our laboratory [56] and in paper I. A similar regulation of SIK2 in response to cAMP has been described in hepatocytes [57] and osteocytes [132]. The effects of insulin on SIK2 phosphorylation and kinase activity diverge between different cells/tissues [57, 64-66] and the regulation of SIK2 in response to insulin in adipocytes has not been elucidated in greater detail. In paper III we investigated effects of insulin on SIK2 with regards to phosphorylation, catalytic and cellular activity and protein stability in adipocytes.

SIK2 is phosphorylated at Thr484 in response to insulin in adipocytes, without altering the catalytic activity

We describe that SIK2 is phosphorylated at a specific residue (Thr484) in response to insulin in adipocytes (human and rat) and that this is not associated with a change in the *in vitro* kinase activity of SIK2. In the presence of an inhibitor of PKB/Akt (MK-2206) the insulin-induced phosphorylation of Thr484 was reduced almost to the basal level suggesting that PKB/Akt, or a kinase downstream of PKB/Akt, is responsible for mediating the major part of this insulin-induced phosphorylation of SIK2 in adipocytes. Compared to Ser343 and Ser358, phosphorylation at Thr484 was observed also in resting cells, when PKB/Akt is inactive, indicating that other kinase(s) are responsible for maintaining this basal phosphorylation. We also observed that the induction in phosphorylation of SIK2 at Thr484 displays a positive correlation to that of PKB/Akt at Ser473, further strengthening the results that the phosphorylation of this site is indeed regulated by the insulin signalling pathway. Our findings on the regulation of SIK2 catalytic activity are consistent with recent observations in hepatocytes where insulin had no effect on the *in vitro* kinase activity of endogenous SIK2 [57]. Moreover, in that study *in vitro* kinase activity of SIK2

isolated from mouse liver did not change in response to refeeding. However, the insulin-induced phosphorylation at Thr484 was not observed in hepatocytes indicating that tissue-specific effects exist [57]. On the other hand, Dentin et al (2007) proposed that insulin activated SIK2 through phosphorylation at Ser358 in HEK293T cells and primary mouse hepatocytes [65]. In our studies we did not detect any induction of Ser358 in response to insulin in adipocytes ([56] and paper III). Insulin-induced activation of SIK2 was also described in retinal glia but the underlying mechanism was not studied [66]. In brown adipocytes, Muraoka et al (2009) instead identified Ser587 to be phosphorylated in response to insulin and this was associated with a reduced cellular activity of SIK2 [64]. Some differences with regards to the insulin-induced changes in SIK2 phosphorylation and activity reported in different cells/tissues are likely explained by the methodology or readout chosen for each respective analysis. Our analysis was directed towards three of the residues that we have previously shown to be phosphorylated in response to cAMP/PKA in adipocytes (Ser343, Ser358 and Thr484) [56], for which we also had access to validated tools (phosphospecific antibodies). Thus, it is still possible that Ser587, or another yet unknown residue, is regulated by insulin in (white) adipocytes.

We have not found evidence that the *in vitro* kinase activity of SIK2 is altered in response to any extra- or intracellular signals ([56] and paper III). In addition to measuring the *in vitro* kinase activity of SIK2 we used the phosphorylation status of CRTC2 and HDAC4, which we characterised as SIK substrates in adipocytes in paper I, as a readout for the *cellular* activity of SIK2. The phosphorylation of HDAC4, but not CRTC2, was increased in response to insulin suggesting that the cellular activity of SIK2 towards specific substrates might be increased after insulin stimulation.

By performing a motif scan of predicted phosphorylation sites in SIK2 using the web-based tool Scansite (http://scansite.mit.edu/), Thr484 is the only site in SIK2 predicted to be phosphorylated by PKB/Akt, arguing against that Ser358 or Ser587 are regulated in response to insulin. However, these sites might instead be phosphorylated by another kinase activated by insulin. In addition to PKB/Akt, Thr484 is predicted to be phosphorylated by PKA (which we have shown in rat and human adipocytes, [56] and paper I) and PKCε. PKCε could thus potentially contribute to the phosphorylation of Thr484, or another residue, in adipocytes. The conserved sites in SIK1 (Thr473) and SIK3 (Thr411) are also predicted to be phosphorylated in a similar way. Previous reports have identified these sites to be phosphorylated by PKA in muscle cells for SIK1 [68] and in adipocytes for SIK3 [59]. The phosphorylation of SIK3 in response to insulin was also analysed using a PKB/Akt consensus motif antibody but SIK3 displayed no sign of insulin-induced phosphorylation [59] in these cells. Whether SIK1 is regulated in a similar manner as SIK2 in response to insulin remains to be elucidated. A similar dual phosphorylation pattern of a single site by PKA and PKB/Akt has also been identified for AMPK [164]

and glycogen synthase kinase 3 (GSK-3) [165]. This crosstalk between different signalling pathways is believed to enable directed control of kinase function depending on the environmental context.

Insulin is important for stabilising SIK2 protein

In paper III we also addressed a potential functional role for Thr484 phosphorylation of SIK2, and hypothesised that it might be important for mediating effects of insulin on SIK protein levels in adipocytes. Previous reports have demonstrated that phosphorylation of SIK2 at Thr484 by CaMKI/IV in neurons [69], or the analogous site in SIK1 (Thr475 in rodents, Thr473 in human) by PKA in muscle cells [68], is involved in the regulation of SIK protein stability. In primary rat adipocytes we observed an increase in SIK2 protein levels after 1-2 hours of insulin treatment and this increase was lost in the presence of the proteasome inhibitor MG132, suggesting that the insulin-induced increase in SIK2 protein levels might involve reduced proteasomal degradation. Additionally, we observed a slight increase in *Sik2* mRNA in primary rat adipocytes after 15-30 minutes of insulin treatment indicating that transcriptional activation might also contribute to the increased SIK2 protein levels. Overnight incubation of primary mouse adipocytes with the protein synthesis inhibitor cycloheximide (CHX) in the absence of insulin markedly reduced SIK2 protein levels, but this decrease was partly prevented in the presence of insulin, further suggesting that insulin promotes SIK2 protein stability. Importantly, SIK3 protein levels were not stabilised by insulin suggesting that the effect is specific to SIK2.

SUMMARY AND CONCLUSIONS

This thesis has addressed the expression, regulation and biological role of SIKs, in particular SIK2, in (human) adipose tissue. In summary, the data presented in this thesis provide important novel insights on SIK function in this tissue. A schematic summary is shown in Figure 11.

In paper I, we demonstrate that the transcriptional regulators CRTC2, CRTC3 and HDAC4 are direct downstream substrates of SIK2 in adipocytes, and can thus be used as a readout for the cellular activity of SIKs in these cells. Moreover, cAMP/PKA-dependent phosphorylation of SIK2 at Ser358 is important for controlling some aspects of its cellular activity towards specific targets. We also show that the regulation of CRTC2 and HDAC4 exist also in human adipocytes, strengthening the physiological relevance of our findings.

In paper II, we determine that SIK2 is the dominant SIK isoform in human adipocytes and demonstrate that the expression of SIK2 and SIK3 is markedly downregulated in adipose tissue from obese or insulin-resistant individuals. This finding was independent of BMI and age for SIK2. Moreover, the expression of SIK2 and SIK3 in adipose tissue is regulated in response to weight change and inflammation (TNF-α). Importantly, our findings on SIK2 expression in human obesity and insulin resistance are in contrast to what was previously identified in obese diabetic (*db/db*) mice [11], and demonstrates that inter-species differences exist with regard to the regulation of SIK2 in metabolic disease. Furthermore, this emphasise the importance to study SIKs in human adipocytes.

From the data presented in papers I, II and IV it is clear that SIKs are involved in promoting glucose uptake in adipocytes and the underlying mechanism(s) involves direct, and positive, effects on the insulin signalling pathway. We also identify a novel regulatory pathway of SIK2 through the specific phosphorylation at Thr484 in response to insulin in adipocytes (paper III). From a functional aspect, insulin stimulation appears to be important to increase SIK2 protein stability.

Taken together, our data suggest that insulin resistance might be a causal factor underlying the downregulation of SIK2 in human adipose tissue. In turn, a reduced SIK2 expression results in impaired insulin action and glucose uptake in adipocytes, which contributes to further exacerbate insulin resistance in these individuals. Given these findings, SIK2 might provide an attractive therapeutic target for the treatment

of metabolic diseases in the future and strategies to promote SIK2 expression or activity would theoretically be beneficial in this context.

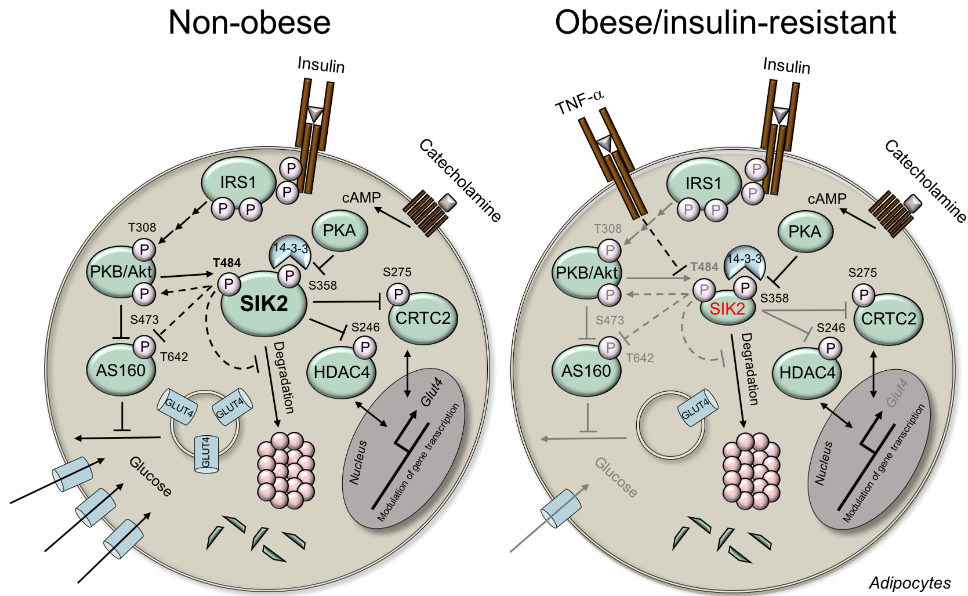

Figure 11. Schematic summary of the main findings in this thesis.
(**Left**) In summary, we have demonstrated that CRTCs (illustrated as CRTC2) and HDAC4 are direct downstream substrates of SIK2 in adipocytes. PKA-mediated phosphorylation at Ser358 results in 14-3-3 binding and is important for the regulation of the interaction between SIK2 and CRTCs. Moreover, we have identified a novel regulatory pathway of SIK2 by insulin in adipocytes. Insulin-induced phosphorylation at Thr484 appears to be important for increasing SIK2 protein stability, likely via reduced degradation by the proteasome. SIKs (illustrated as SIK2) are important for promoting glucose uptake in adipocytes, likely via direct effects on the insulin signalling pathway that promote phosphorylation of PKB/Akt at Thr308 and Ser473, and AS160 at Thr642, and GLUT4 translocation to the plasma membrane.However, the exact underlying mechanism has not been elucidated in detail. In 3T3-L1 and SIK2 KO mouse adipocytes SIK2 also promote GLUT4 expression through CRTC2/HDAC4-dependent regulation of gene transcription. (**Right**) In obese or insulin-resistant humans, SIK2 expression is markedly downregulated (red) and the insulin-induced phosphorylation at Thr484 is impaired. The mechanism underlying a reduced SIK2 expression is suggested to involve direct effects of TNF-α on SIK2 protein expression and an impaired insulin-mediated stabilisation of SIK2 protein levels. Mimicking reduced SIK expression/activity in human adipocytes results in reduced phosphorylation of PKB/Akt and AS160, and a reduced glucose uptake. Grey arrows and text illustrate impaired processes, and dashed arrows indicate that the primary mechanism is not known.

FUTURE PERSPECTIVES

This thesis has shed light on SIK function in human adipose tissue, an area that was previously unexplored. The findings in this thesis have contributed both to gaining novel insights into the function of SIKs in adipocytes, and provided a piece of the puzzle to the mechanisms underlying metabolic diseases such as obesity and T2D. However, there are still questions that remain unanswered and warrant further studies.

We identified that the expression of SIK2 and SIK3 was markedly downregulated in adipose tissue from obese or insulin-resistant humans and that SIK expression was regulated in response to weight loss and inflammation. However, we were not able to elucidate in great detail whether the downregulation of SIKs is a cause or consequence of metabolic disease. In order to identify factors underlying the regulation of SIK expression it would be interesting to expand the investigation to include humans with T2D. At a molecular level, it would also be interesting to examine if SIK expression is regulated by other inflammatory cytokines than TNF-α, as well as if there is a correlation between SIK and TNF-α expression in the adipose tissue *per se*.

The strong correlation between SIK2 expression and systemic insulin resistance, suggest that a preserved insulin action might be important for controlling SIK2 expression. Interestingly, we identified that insulin specifically induced phosphorylation of SIK2 at Thr484 and insulin stimulation was important to increase SIK2 protein stability. Studies in other tissues have demonstrated that glucose or insulin might play a role in regulating SIK2/SIK3 expression both on transcriptional and post-translational levels [66, 111, 162]. In future studies, it would thus be interesting to analyse if the expression of SIK2 and SIK3 is regulated in response to insulin and/or glucose in *human* adipocytes. Moreover, the requirement of Thr484 phosphorylation for the regulation of SIK2 protein stability, or other potential functional effects, needs to be further investigated.

We also demonstrate that SIKs promote glucose uptake in adipocytes, via direct effects on the insulin signalling pathway. In future studies, it will be important to pinpoint at which level(s) in the insulin signalling pathway that SIKs act in human adipocytes. Several studies point towards a role for SIK2 in the regulation of insulin signalling. Modulation of SIK2 has been reported to influence the phosphorylation of PKB/Akt [66, 75, 112], but whether PKB/Akt was the primary target was not

investigated in those studies. Additionally, IRS [11, 66] and the PI3K regulatory subunit p85 [81] have been suggested to be SIK2 substrates by *in vitro* phosphorylation assays. So far, we have attempted to address the role for SIKs in regulating IRS1 phosphorylation in primary adipocytes using phosphospecific antibodies directed towards known regulatory sites. However, it is known that IRS1 is phosphorylated on multiple residues [22] and the risk with focused analyses is that the true regulated phosphorylation sites are missed due to the fact that they are either novel, or that there are no commercially available tools to study them. In order to conclude on the role of SIK2 for IRS1 and p85 phosphorylation it would be important to perform an unbiased analysis of phosphorylation sites regulated by SIK2 *in vitro*, and in adipocytes, using mass spectrometry. An *in vitro* phosphorylation assay would also be informative in order to evaluate the properties of IRS1 and p85 as SIK2 substrates compared to the validated CRTCs and HDACs.

Finally, data from available global SIK2 KO mouse models have not been conclusive with regard to metabolic phenotypes possibly as a result of embryonic deletion and functional redundancy among SIK isoforms. In the future, it would be interesting to generate inducible and/or adipose tissue-specific SIK2 KO mice to study effects of SIK2 in adult mice and in this tissue alone.

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