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2012

[Link to publication](#)

Citation for published version (APA):

Henriksson, E. (2012). *LKB1 signaling pathways in adipocytes - Focus on the AMPK-related kinase SIK2*. [Doctoral Thesis (compilation), Department of Experimental Medical Science]. Department of Experimental Medical Science, Lund University.

Total number of authors:

1

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LKB1 signaling pathways in adipocytes

Focus on the AMPK-related kinase SIK2

Emma Henriksson



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December 7th 2012 at 9.00

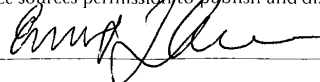
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Organization LUND UNIVERSITY		Document name DOCTORAL DISSERTATION	
Medical Faculty Department of Experimental Medical Science Division of Diabetes, Metabolism and Endocrinology		Date of issue December 7th 2012	
Author(s) Emma Henriksson		Sponsoring organization	
Title and subtitle LKB1 signaling pathways in adipocytes - Focus on the AMPK-related kinase SIK2			
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Key words: Adipocyte, signal transduction, protein phosphorylation LKB1, AMPK, SIK2, SIK3, cAMP			
Classification system and/or index termes (if any):			
Supplementary bibliographical information:		Language	
ISSN and key title: 1652-8220		ISBN 978-91-87189-57-9	
Recipient's notes		Number of pages 155	Price
		Security classification	

Distribution by (name and address)

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LKB1 signaling pathways in adipocytes

Focus on the AMPK-related kinase SIK2

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The Cover

Confocal image of 3T3-L1 adipocytes taken by the author



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Lund University, Faculty of Medicine Doctoral Dissertation Series 2012:94
ISBN 978-91-87189-57-9
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2012

To my Family

“The only true wisdom is in knowing you know nothing”

Socrates

"It's SIK"

Daniel Eklund

Abstract

Adipose tissue does not only store and release energy in response to hormones, it is also known as an endocrine organ, secreting important factors and hormones that influence for example appetite and insulin sensitivity. The association of type 2 diabetes with obesity has been known for quite some time. Understanding the cellular mechanisms of adipocyte function is of great importance in understanding when and why excess adipose tissue becomes dangerous. Signal transduction pathways used for example by hormones to control cellular function, often consists of protein kinases. These enzymes constitute a large part of our genome and are crucial for the regulation of almost all cellular processes. AMP-activated protein kinase (AMPK) is known for its various roles in the regulation of metabolism and is activated when cellular energy levels are low, which is reflected in increased levels of AMP. In addition to AMPK, some of its related kinases, including the salt-inducible kinases (SIKs), have also been implicated in the regulation of metabolism. LKB1 is known as a tumor suppressor and was recently found to be required for the activity of AMPK and most of its related kinases, phosphorylating a specific threonine residue in their activation loop. The aim of this thesis was to investigate the regulation of LKB1 signaling pathways in adipocytes, with a focus on SIK2, which is of particulate interest in adipocytes due to its high abundance in these cells. We show that AMPK activity is regulated by LKB1 and CaMKK in adipocytes and describe a regulation of SIK2 and SIK3 by cAMP/PKA signaling. The PKA-dependent phosphorylations of SIK2 and SIK3 were identified and shown to mediate a binding to 14-3-3 proteins, resulting in a re-localization from a particulate fraction to the cytosol, and a decrease in activity, respectively. In addition, we suggest that the transcriptional regulators CREB-regulated transcription co-activator (CRTC) 2, -3 and histone deacetylase (HDAC) 4, are substrates of SIK2 in adipocytes. Based on our findings, we hypothesize that SIK isoforms take part in transcriptional regulation of genes involved in lipid and glucose metabolism in adipocytes, through their action on HDAC4, CRTC2 and CRTC3, and potentially also other transcriptional regulators. We also identified PP2A as an interacting partner of SIK2 in adipocytes and future studies will further evaluate the importance and function of this interaction.

In conclusion, this thesis has revealed regulation of AMPK, SIK2 and SIK3, important for adipocyte function and provided preliminary data connecting SIK2 to both lipid and glucose metabolism in adipocytes.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Vår fettväv är mer än bara några extra kilon

I dag har extrem övervikt och dess följsjukdomar, som t.ex. diabetes, blivit allt vanligare. Typ 2 diabetes, eller åldersdiabetes, är en komplex sjukdom där många faktorer ligger till grund för utvecklandet av det tillstånd som är mest karakteristiskt för diabetes, nämligen högt blodsocker. Högt blodsocker kan bero på kroppens oförmåga att producera insulin, ett hormon som är avgörande för att vävnader så som muskel, fett och lever ska kunna ta upp energi i form av socker (glukos) och/eller lagra den i form kolhydrat och fett efter en måltid. En okänslighet mot insulin i vävnader som muskel och lever, så kallad insulinresistens, leder också till ökade nivåer av socker i blodet och anses vara en underliggande orsak till utveckling av typ 2 diabetes. Insulinresistens, eller brist på insulin, skapar problem eftersom levern fortsätter med egenproduktionen av socker i en process kallad glukoneogenes trots att blodsockernivån är hög och fettväven frisätter energi i form av fria fettsyror, genom en process som kallas lipolys. Detta leder till ytterliggare förhöjda blodsockernivåer och andra oönskade effekter som höga nivåer av fria fettsyror i blodet.

Det finns en stark koppling mellan övervikt och typ 2 diabetes, då hela 80 % av alla typ 2 diabetiker är överviktiga. En orsak till att vissa överviktiga utvecklar diabetes verkar vara att fettväven hos dessa individer fungerar dåligt. Förutom fettvävens funktion som mekaniskt skydd för organ och som plats för lagring och frisättning av energi, så frisätter den också hormoner som visats vara av vikt för insulinkänsligheten i andra vävnader. Ett sätt att öka förståelsen och kunskapen kring fetma och diabetes är att på detaljnivå klargöra hur fettceller fungerar och hur deras funktion styrs av hormoner via så kallad cellsignalering. Cellsignalering är en form av kommunikation inne i cellen, som utförs under ett slags organiserat kaos. I de studier som denna avhandling baseras på har vi studerat ett proteinkinase vid namn LKB1 och dess signalvägar i fettceller.

Proteinkinaser – ett sändebud i cellen

Ett proteinkinase är ett enzym och fungerar som ett sändebud i cellen. Proteinkinaser är avgörande för att cellers funktioner ska kunna styras av signaler utifrån, t.ex. hormoner, genom att det hjälper till att vidareförmedla signalerna inne i cellen. Proteinkinaser är den största familjen av enzymer i vår arvs massa. Från ett kemiskt perspektiv så sätter proteinkinaser på en fosfatgrupp på andra proteiner genom en process som kallas proteinfosforylering. Fosfatgruppen utgör en signal för proteinet, som därmed kan aktiveras eller stängas av. Med andra ord är proteinfosforylering ett av språken som används inne i cellen för att kommunicera. En proteinfosforylering kan också fungera som en adresslapp, vilket resulterar i en förflyttning av proteiner, men kan också leda till att proteiner binder till varandra eller skiljs åt. Alla dessa sätt är mekanismer som cellen använder för att på rätt sätt svara på de signaler kroppen skickar. Proteinkinaser är av stort intresse i läkemedelsindustrin och läkemedel riktade mot specifika proteinkinaser används redan för att behandla till exempel cancer.

LKB1 – en ordningsvakt mot cancer med koppling till ämnesomsättning.

LKB1 är ett proteinkinase som visats vara involverat i utvecklingen av en viss cancerform, kallad Peutz-Jeghers syndrom och som ger ökad risk för bildandet av tumörer i mag-tarmkanalen. Personer med avvikelser i den gen som kodar för LKB1 har lättare för att utveckla cancer. Man kan säga att om LKB1 inte finns, så fungerar inte vissa av cellens säkerhetsprogram som normalt stoppar celler med avvikande beteende, så som cancerceller. Genom att störa dessa säkerhetsprogram så ökar därför risken för cancerutveckling. För några år sedan upptäckte man att LKB1 krävdes för att ett annat kinas, AMP-aktiverat proteinkinase (AMPK) och flera av dess besläktade kinaser, skulle fungera. LKB1 fosforylerar nämligen en viktig del av AMPK som kallas aktiveringsloop. En specifik fosforylering på aktiveringsloopen är avgörande för ett proteinkinase ska fungera och kan likställas en ON-knapp. LKB1 slår alltså på ON-knappen för AMPK. Vad som är intressant med upptäckten att LKB1 var viktig för AMPK, är att detta utgjorde en koppling mellan cancer (LKB1) och ämnesomsättning (AMPK). AMPK är nämligen välkänt i sammanhang som rör vår ämnesomsättning och deltar i energiregulering både på cellnivå och kroppsnivå.

AMPK – en räddare i nöden

AMPK känner av låga energinivåer i cellen och ser till att energin återställs genom att sätta igång processer som bildar energi, men också genom att stänga av processer som kostar energi. Om AMPK fanns i ett batteri skulle det se till att batteriet aldrig blev urladdat. Energi finns tillgänglig i en cell i form av en molekyl som heter ATP. Vid energikonsumtion omvandlas denna molekyl stegvis till AMP. AMPK känner av höga nivåer av AMP (låg energinivå), vilket leder till en aktivering av kinaset i flera av de vävnader som är viktiga i vår ämnesomsättning. AMPK har mest studerats i lever och muskel och verkar aktiveras av metformin, ett av de vanligaste läkemedlen vid behandling av typ 2 diabetes. I fettväven har man föreslagit flera viktiga funktioner för AMPK, men AMPK är egentligen mest känt för sin roll i lever och muskel.

SIK2 och SIK3 i vår fettväv – vilka är ni och vad vill ni?

Två familjemedlemmar till AMPK som kallas SIK2 och -3, är relativt ostuderade, men har på grund av deras släktskap med AMPK också föreslagits vara involverade i ämnesomsättningen. Hittills finns dock inga starka bevis för att SIK2 och -3 känner av låga energinivåer på det sättet som AMPK gör. Andra roller i ämnesomsättningen har föreslagits, speciellt för SIK2, men den senaste tiden också för SIK3. Det finns dock stora luckor i kunskapen kring hur dessa proteinkinaser regleras, till exempel av hormoner, och speciellt kring deras roll i fettväven. Något som gör SIK2 extra intressant är att det är rikligt förekommande just i fettväven, vilket föreslår att det har en viktig roll där. Ett sätt att få ledtrådar om vilken roll ett proteinkinaset har är att studera dess reglering. Genom att studera reglering av ett protein (i vårt fall AMPK, SIK2 och -3) kan man få reda på vilka hormoner eller andra yttre signaler som påverkar t.ex. dess nivåer eller aktivitet. Detta gör i sin tur att man kan koppla proteinet till kända effekter av just det hormonet på den vävnad man studerar (i detta fall fettväv).

LKB1, men också CaMKK, som svar på ett hormon från sköldkörteln trycker på ”ON-knappen” hos AMPK i fettceller

Genom de studier som ingår i denna avhandling har vi ökat kunskapen om vilka signaler och hormoner som reglerar AMPK (Artikel I), SIK2 (Artikel II) och SIK3 (Artikel III) i fettväv. I Artikel I visar vi att LKB1 är viktigt för att AMPK ska vara aktivt i fettväv, men också att AMPK kan aktiveras av ett hormon från sköldkörteln (tyreoideahormon, T_3). I denna studie tog vi också reda på vilket protein det är som trycker på ON-knappen som svar på sköldkörtelhormonet. Våra resultat tyder på att detta är ett proteinkinase vid namn CaMKK. Därmed stämmer våra resultat överens med tidigare studier som föreslagit att CaMKK kan sätta igång AMPK i andra vävnader. SIK2 och SIK3 verkar däremot inte aktiveras av CaMKK på ett liknande sätt.

SIK2 och SIK3 deltar i det organiserade kaoset inne i fettcellen som svar på katekolaminer

I två andra studier som presenteras i avhandlingen (Artikel II och Artikel III) visar vi att katekolaminer, hormon som frisätts t.ex. vid låga blodsockernivåer och som signalerar för nedbrytning av fett i fettcellen, reglerar SIK2 och SIK3. Man skulle kunna säga att våra studier visar att SIK2 och SIK3 är delaktiga i kommunikationen, det organiserade kaoset i cellen, som följer efter att cellen tagit emot en signal från katekolaminer, t.ex. adrenalin. Katekolaminer leder till ökad aktivitet av, med andra ord sätter igång, ett proteinkinase vid namn protein kinas A (PKA), som i sin tur fosforylerar SIK2 och SIK3. För att bättre förstå denna process bestämde vi oss för att ta reda på vilken specifik aminosyra (byggstenarna i ett protein) som fosforyleras av PKA. För SIK2 fanns det en specifik aminosyra som verkar avgörande (serin 358), medan för SIK3 så fanns det flera stycken (treonin 469, serin 551 och serin 674) som var viktiga för fosforyleringen vi påvisade som svar på katekolaminer. Genom att identifiera vilka aminosyror som är inblandade skulle vi senare kunna koppla dessa fosforyleringar till potentiella funktioner hos kinaserna. PKA-fosforyleringen av SIK2 och SIK3 ledde till en interaktion med ett protein vid namn 14-3-3. 14-3-3 binder över 300 proteiner i cellen och fungerar ofta som en upphängningsanordning i den del av cellen som kallas cytosol. Fosforylering av SIK3 och inbinding till 14-3-3 i fettceller minskar aktiviteten av SIK3, men verkar inte påverka var i cellen SIK3 befinner sig.

Fortsatta studier krävs dock för att bekräfta detta. För SIK2 var situationen den omvända, inbindning till 14-3-3 förändrade inte aktiviteten, men ledde till en ansamling av SIK2 i cytosol-delen av cellen, vilket stämmer överens med 14-3-3 proteinernas kända funktion. Även om aktiviteten inte förändrades så kan detta i teorin innebära att SIK2 göms undan från sitt mål i cellen, vilket i sin tur kan resultera i en nedsatt funktion av SIK2.

SIK2 – delaktigt i produktion av proteiner viktiga för fettcellens funktion genom att styra avskrivning av gener?

I Artikel IV utgick vi från våra resultat i Artikel II, där vi visade att katekolaminer påverkar SIK2 på flera nivåer, för att hitta substrat till SIK2. Med substrat, eller målprotein, menas i detta fall ett protein som utsätts för fosforylering av SIK2 vilket för signalen vidare i cellen. Två grupper av proteiner som kallas CRTCs och klass II HDACs påverkar avskrivningen av gener (vår arvs massa) på olika sätt. Detta kallas genreglering och kan leda till ökade eller minskade nivåer av enzymer eller andra proteiner som deltar i viktiga cellulära processer. CRTCs och klass II HDACs har tidigare föreslagits vara substrat till SIK2 och regleras också av katekolaminer. I Artikel IV föreslår vi att vissa av dessa (CRTC2, CRTC3 och HDAC4) är målprotein för SIK2, och att SIK2 på så sätt reglerar avskrivning av gener i fettceller. Ett sätt vi visar detta på är genom att på experimentell väg förändra nivåerna av SIK2 i fettceller, och därefter påvisa att fosforylering av de föreslagna målproteinerna därmed också förändras. Ett annat intressant fynd i denna studie är att om man ökar mängden SIK2 i fettceller ökar också upptaget av socker i cellerna. Vi spekulerar i om det är möjligt att SIK2 är involverad i avskrivningen av gener som är viktiga för upptaget av socker i fettceller. Vi har också förändrat nivåerna av SIK2 i fettcellen för att studera effekter av detta på fettnedbrytning och frigörande av fettsyror. Eftersom fettväven frigör fettsyror i närvaro av katekolaminer, utgör detta en intressant koppling till den reglering av SIK2 och SIK3 som svar på katekolaminer som vi beskrivit i Artikel II och III. Fortsatta studier får vidare klargöra precis vilken roll SIK2 har i fettcellen.

Fettcellens snicke-snack är viktigt för vårt välmående

Med ökade kunskaper kring cellsignaleringens deltagare i friska och sjuka celler kan man bättre förebygga och behandla sjukdomar, så som typ 2 diabetes. Förmågan hos en cell att ta emot, samt att korrekt svara på en signal utifrån är avgörande för kroppens normala funktion. På lång sikt kan en ökad förståelse kring fettcellens funktioner, så som lagring och frigörande av energi, och de signalmolekyler, t.ex. proteinkinaser, som deltar i cellsignaleringen och därmed regleringen av dessa funktioner användas för utveckling av nya läkemedel.

LIST OF PAPERS

Papers included in this thesis

- I. **Regulation of AMP-activated protein kinase by LKB1 and CaMKK in adipocytes.**
Gormand A., Henriksson E., Ström K., Jensen T., Sakamoto K., Göransson O. *2011 J Cell Biochem.* 112:1364-75

- II. **The AMPK-related kinase SIK2 is regulated by cAMP via phosphorylation at Ser358 in adipocytes.**
Henriksson E., Jones H.A., Patel K., Peggie M., Morrice N., Sakamoto K., Göransson O. *2012 Biochem. J.* 444:503-14

- III. **cAMP-elevation mediated by β -adrenergic stimulation inhibits salt-inducible kinase (SIK) 3 activity in adipocytes.**
Berggreen C., Henriksson E., Jones H.A., Morrice N., Göransson O. *2012 Cell Signal.* 24:1863-71

- IV. **Identification of cAMP-responsive molecular targets of SIK2 in adipocytes**
Henriksson E., Säll J., Gormand A., Stenkula K., Göransson O.
Manuscript

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Papers not included in this thesis

Rose hip exerts antidiabetic effects via a mechanism involving downregulation of the hepatic lipogenic program

Andersson U., Henriksson E., Ström K., Alenfall J., Göransson O., Holm C.
2011 Am J Physiol Endocrinol Metab. 300:E111-21

ABBREVIATIONS

ACC	Acetyl-CoA Carboxylase
ACREB	Dominant negative CREB
AICAR	5-amino-4-imidazolecarboxamide ribonucleoside
ALK5	TGF β type I receptor/ALK5
AMPK	AMP-activated protein kinase
aPKC	atypical Protein kinase C
ATGL	Adipose triglyceride lipase
BRSK	Brain-specific kinase
CaMK	Ca ²⁺ /Calmodulin kinase
CaMKK	Ca ²⁺ /Calmodulin kinase kinase
CAR	Coxsackievirus and adenovirus receptor-expressing
CBM	Carbohydrate-Binding Modules
CID	Collision-induced dissociation
ChREBP	Carbohydrate-responsive element-binding protein
CRE	cAMP-responsive elements
CREB	CRE-binding protein
CRTC	CREB-regulated transcription co-activator
dSIK	Drosophila SIK
DSP	Dithiobis succinimidyl propionate
ESI	Electrospray ionization
FA	Fatty acid
FAS	Fatty acid synthase
GSK β	Glycogen synthase kinase 3 β
HAT	Histone acetyltransferas
HDAC	Histone deacetylase
HEK293	Human embryonic kidney
HFD	High fat diet
HSL	Hormone-sensitive lipase
IRS1	Insulin receptors substrate
LC-MS/MS	liquid chromatography – tandem - mass spectrometry
LPL	Lipoprotein lipase
LKB1 ^{hypo}	LKB1 hypomorphic flox/flox
MARK	Microtubule affinity-regulating kinase
MELK	Maternal embryonic leucine zipper kinase
MO25	Mouse protein 25
NLS	Nuclear localization signal

OA	Okadaic acid
OK	Opossum kidney
PGC1 α	PPAR γ co-activator-1 α
PKA	Protein kinase A
PKB	Protein kinase B
PP2A cat	Protein phosphatase 2A catalytic subunit
PP2A reg	Protein phosphatase 2A regulatory subunit
PP1B	Protein phosphatase 1B
PME1	Protein phosphatase methylesterase-1
PPAR	Peroxisome proliferator-activated receptor
SIK	Salt-inducible kinase
SREBP1	Sterol regulatory element-binding protein 1
STRAD	STE20-related adaptor
T2D	Type 2 diabetes
TAG	Triacylglycerol
TAO1	Thousand and one amino acids)
T-loop	Activation loop
UBA	Ubiquitin associated
WAT	White adipose tissue

GENERAL INTRODUCTION

Diabetes is a group of metabolic diseases with the common characteristic of high blood glucose, due to the inability of the pancreas to produce sufficient amounts of insulin, or because target tissues have a reduced ability to respond to the hormone. The latter is in other terms described as insulin resistance, and is common in type 2 diabetes (T2D), whereas T1D is mainly characterized by a reduced production of insulin. Insulin resistance means that more insulin is needed to achieve the same effects as in insulin sensitive subjects. In the development of the disease, insulin resistance is compensated for by increased insulin production [1]. One theory is that, over time, this increased demand of insulin will lead to exhaustion of the insulin producing β -cells, eventually leading to cell death and deficiency of insulin production and secretion. More than 80% of all T2D patients are over-weight, demonstrating a strong connection between obesity and insulin resistance. Insulin is not only important to regulate plasma glucose levels, but also plays a role in the regulation of lipid metabolism. Adipose tissue stores energy in the form of triglycerides and releases energy in the form of fatty acids via a process called lipolysis. One of the important functions of insulin in adipose tissue is to inhibit lipolysis and thereby promote lipid storage. If adipose tissue becomes insulin resistant, there is an increased release of fatty acids from the triglyceride stores, resulting in harmful levels of lipids in the blood [2, 3]. In addition, adipose tissue plays a crucial role in whole body energy homeostasis, not only via its vital functions in energy storage and fat mobilization, but also as an endocrine organ. Circulating factors that are secreted from adipose tissue can have multiple effects, including influencing appetite and the insulin sensitivity in other tissues [4].

Protein kinases are important in regulating cellular function and constitute a large proportion of our genome. The human kinome, a categorization of protein kinases, based on the sequence homology of their kinase domains, was presented in 2002 [5]. Protein kinases catalyze the protein phosphorylation reaction, one of the major mechanisms for the transduction of intracellular signals. Protein phosphorylation increases or decreases enzyme activity through conformational changes, but can also lead to the creation of recognition sites for other proteins. Lately, protein kinases have been referred to as “drug targets of the future” [6]. A very interesting kinase from a metabolic point of view is AMP-activated protein kinase (AMPK). AMPK is an energy sensor, which when activated by AMP favors energy producing pathways and shuts down energy consuming processes [7, 8]. AMPK is not only involved in cellular energy metabolism, but also regulates whole body energy homeostasis, by responding to different hormonal cues. Drugs used in the

treatment of T2D, such as metformin (inhibition of hepatic glucose production) and thiazolidinediones (insulin sensitizers) are suggested to activate AMPK [9, 10]. Important roles for AMPK have been described especially in muscle and liver. Characterizing the function of AMPK in adipocytes is also of importance, in order to understand how AMPK contributes to the regulation of whole body energy metabolism. The kinase domain of AMPK shares homology with a number of AMPK-related kinases, which are all regulated by the common upstream kinase LKB1. Interestingly, AMPK-related kinases have also been implicated in the regulation of energy metabolism. MARK2, -3 or 4-deficient mice all show signs of resistance to diet induced obesity [11-13]. Deletion of *Nuak1* in muscle improves glucose homeostasis and protects against high fat diet induced insulin resistance [14]. Lately, SIK3-deficient mice were presented with cholestasis and altered glucose- and lipid metabolism [15]. Although SIK2-deficient mice did not show any significant metabolic phenotype (as reported so far [16, 17]), the abundant expression of SIK2 in adipocytes and earlier suggested roles for SIK2 in glucose and lipid metabolism makes it an interesting AMPK-related kinase to study from a metabolic perspective [18-21]. In this thesis, the regulation of LKB1 signaling pathways in adipocytes, with a focus on SIK2, was investigated.

BACKGROUND

White adipose tissue

The white adipose tissue (WAT) is our main place for storage of energy and takes part in the critical maintenance of whole body energy homeostasis. Adipose tissue is mainly made up by adipocytes - cells specialized in storing triacylglycerols (TAGs). Adipose tissue is also composed of endothelial cells, fibroblasts and macrophages - all contributing to the secretory function of the tissue. In the fed state, white adipocytes have a great capacity to store lipids in the form of TAGs. Upon food restriction or when other tissues demand energy, such as during exercise, TAGs are released from adipose tissue in the form of glycerol and fatty acids (FAs) [22].

Lipoprotein lipase (LPL) is produced in adipocytes and exported to the vascular endothelium where it hydrolyzes TAGs. It plays a crucial role, since TAGs from the circulation constitute the majority of the TAGs stored in adipocytes. A *de novo* lipogenesis, in other words the production of fatty acids from other substrates such as glucose, is used to a much smaller extent in humans (Figure 1). The fatty acids that enter the adipocytes are re-esterified to form TAG for storage in the lipid droplet (Figure 1). In the post-prandial state, insulin promotes the storage of lipids by inducing LPL expression and activity, GLUT4 translocation to the plasma membrane, thereby facilitating glucose uptake, as well as the esterification of fatty acids. Insulin also stimulates *de novo* synthesis of fatty acids by inducing the activation and/or expression of lipogenic proteins such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) [23-25]. In addition, insulin also negatively regulates lipolysis (described below) to favor lipid storage. Protein kinase B (PKB) has been shown to be critical for many, if not most of the effects of insulin on glucose- and lipid metabolism in adipocytes. The lipid droplet of adipocytes is coated with a protein called perilipin. The regulation of perilipin is important in accessing the lipids stored in times of high energy demand or in response to fasting. TAGs are hydrolyzed into fatty acids and glycerol in a process called lipolysis (Figure 1). Lipolysis is stimulated by catecholamines, such as adrenergic hormones, which are produced either by the adrenal medulla or by the local sympathetic innervation. These hormones signal via an increase in cAMP levels and the activity of protein kinase A (PKA). Perilipin is phosphorylated in response to increased cAMP levels, allowing hormone sensitive lipase (HSL) to access the TAGs [26, 27]. HSL is one of the key enzymes in lipolysis and is activated and

translocated from the cytosol to the lipid droplet as a result of reversible PKA phosphorylation of several sites [28, 29]. Recently, adipose triglyceride lipase (ATGL) was also identified and found to be important, especially for basal lipolysis [30]. In addition to these two essential lipases, a third lipase is also present, monoglyceride lipase [31].

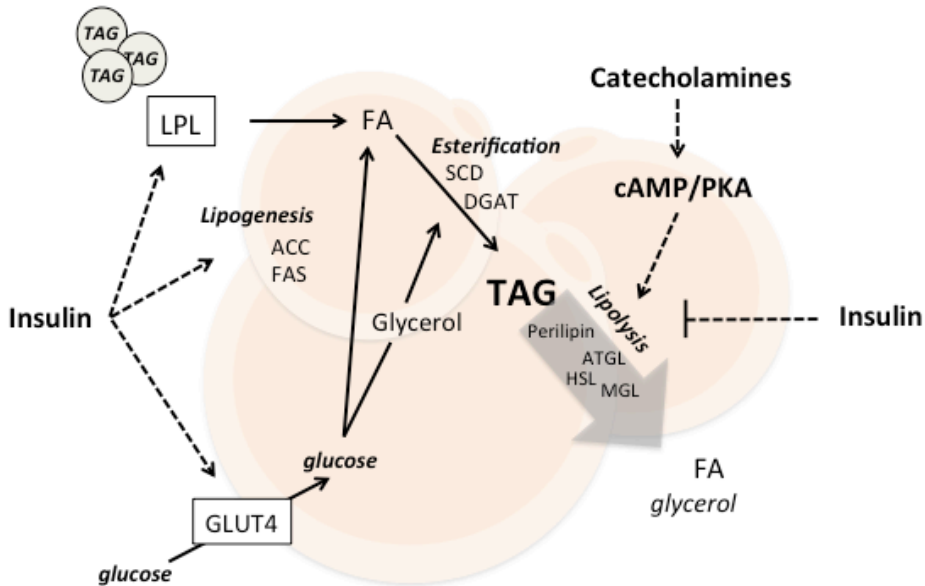


Figure 1 Overview of lipid and glucose metabolism in white adipose tissue

Energy is stored in the adipocyte as triacylglycerol (TAG). Accumulation of TAG is promoted by insulin as indicated by dashed arrows. Lipoprotein lipase (LPL) hydrolyzes TAG in circulating lipoproteins and thereby provides the adipocyte with fatty acids (FA) for esterification. The glucose transporter GLUT4 delivers glucose to the adipocyte for *de novo* lipogenesis of FA and glucose also provides glycerol for the esterification of FA. Important enzymes are indicated; Acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD), diacylglycerol acyltransferase (DGAT). Insulin further promotes lipid accumulation by inhibiting lipolysis. Hydrolysis of lipids in the process known as lipolysis is stimulated by catecholamines and cAMP/PKA signaling as indicated. Lipolysis provides FA for use as energy substrate by other tissues. Perilipin coats and thereby regulate the access to the lipid droplet. Adipose triglyceride lipase (ATGL), hormones sensitive lipase (HSL) and monoglyceride lipase (MGL) all participate in the hydrolysis of TAG. Inspired by Frayn 2010 [22].

In addition to its function in energy storage and release, adipose tissue is an endocrine organ. Hormones or factors produced by adipose tissue are also called adipokines, and two of the most important examples include leptin and adiponectin [32-34]. Leptin action in the hypothalamus reduces food intake and is produced in proportion to the amount of adipose tissue present. Other roles of leptin include stimulation of fatty acid oxidation in muscle and liver, and this is believed to at least partly be mediated via AMPK activation [35]. The plasma concentration of adiponectin and its secretion is reduced with increased adipose tissue [36]. Adiponectin acts on muscle and liver, inducing fatty acid oxidation in both, activating muscle glucose utilization and decreasing the hepatic glucose production [37, 38]. It has been suggested that adiponectin requires AMPK for its effects in liver [39].

LKB1 as a master regulator

LKB1 is a highly conserved serine-threonine kinase and was originally identified as the tumor suppressor responsible for an inherited susceptibility to a form of cancer named Peutz-Jegher's syndrome [40, 41]. Humans with Peutz-Jegher's syndrome are heterozygous for loss-of-function mutations in the LKB1 gene (*STK11*). The LKB1 gene is also frequently mutated in spontaneous cancers especially in non-small cell lung carcinoma and also in the cervical cancer from the patient Henrietta Lacks and thereby the HeLa cell line [42]. LKB1 is dependent on a complex formation with two accessory proteins, STE20-related adaptor (STRAD) and mouse protein 25 (MO25), for its activity [43-45]. Unlike the majority of protein kinases, LKB1 is activated in a phosphorylation-independent manner, probably via the binding to STRAD and MO25. The non-catalytical N- and C- terminal regions of LKB1 are not related to any other proteins and do not contain any known domain structures [46]. Crystallization of the LKB1/STRAD/MO25 complex suggested that the role of STRAD is to promote the interaction between MO25 and LKB1 [47, 48]. The importance of LKB1 in the regulation of several crucial cellular processes, including angiogenesis, cell cycle arrest, cell polarity and energy metabolism has been established for example by the use of several different transgenic LKB1 models [46]. With this wide array of cellular functions, it is not surprising that deletion of LKB1 in mice leads to embryonic lethality. Interestingly, LKB1 was identified as the major upstream kinase of AMPK and 11 of its 12 related kinases and is required for their kinase activity [43, 49-51]. This suggests that these kinases might mediate some of the functions described for LKB1 and also opened up for a potential role of LKB1 in metabolism.

Although LKB1 is required for the effects of AMP on AMPK, LKB1 is believed to be constitutively active and does not appear to respond to conditions known to activate AMPK [43, 49-53]. LKB1 is phosphorylated on several residues, for example by PKA and ATM/ATR, however these phosphorylations do not significantly influence LKB1 activity *in vitro* [54, 55]. Nevertheless, it has been reported that phosphorylation of LKB1 on Thr336 is important for the ability of LKB1 to inhibit cell growth [55, 56] and PKA phosphorylation of LKB1 on Ser431 was suggested to be important for cell polarity [57]. The underlying mechanisms for these effects of phosphorylation are not entirely clear, but one suggestion is that phosphorylation influences LKB1 function by affecting its cellular localization. When LKB1 is bound to STRAD and MO25, it is localized to the cytoplasm, representing an active complex [44, 45]. Recent studies suggest that 14-3-3 proteins could interact with phosphorylated LKB1, thereby interfering with the ability of LKB1 to interact with STRAD and/or with its substrates [58, 59].

AMP-activated protein kinase (AMPK) is a sensor of energy status

ATP is the immediate source of energy in the cell and intracellular ATP concentration needs to be maintained at adequate levels to ensure fundamental cellular functions. AMPK is often called an energy sensor, because it is activated in response to low energy levels, and favors the production of ATP via phosphorylation of a broad range of downstream targets in the cell [8]. The major role of AMPK is thus to maintain cellular energy homeostasis by switching on catabolic pathways like glucose uptake, glycolysis, fatty acid uptake and fatty acid oxidation, while switching off anabolic pathways like fatty acid synthesis, triglyceride synthesis, protein synthesis and glycogen synthesis [60-64]. In addition to energy stress, numerous hormones and/or cytokines also modulate AMPK, leading to a regulation of energy balance at the whole body level. Many of these agents modulate AMPK in the hypothalamus, including leptin and adiponectin [61, 65].

Structure and regulation of AMPK by AMP and ADP

AMPK is a heterotrimeric protein, consisting of a catalytic α -subunit ($\alpha 1$ or $\alpha 2$), and two regulatory subunits, β ($\beta 1$ or $\beta 2$) and γ ($\gamma 1$, $\gamma 2$ or $\gamma 3$). In mammals, there are seven genes encoding AMPK, one for each isoform of the subunits [8]. Although there might be some preferred combinations, all 12 heterotrimeric

combinations can be formed. The catalytic $\alpha 2$ subunit is the main isoform found in heart and muscle, liver cells express both $\alpha 1$ and $\alpha 2$ isoforms and in WAT $\alpha 1$ is the predominant isoform [66-68].

The catalytic α -subunit contains the serine/threonine kinase domain at the N-terminus including the activation loop (T-loop) with a conserved threonine residue, Thr172 in human AMPK $\alpha 1$ [69]. The phosphorylation of this residue by LKB1 and other suggested upstream kinases is absolutely essential for its intrinsic kinase activity. The function of the β -subunit seems to be as a core for the heterotrimeric complex. Via the C-terminal part of the β -subunit, the C-terminal domain of the α -subunit is connected to the N-terminal region of the γ -subunit [70, 71]. The β -subunit also contains carbohydrate-binding-modules (CBMs), which are non-catalytic domains suggested to induce binding of AMPK to glycogen in intact cells [72, 73]. The γ -subunit contains CBS motifs, representing binding sites for nucleotides, such as AMP [74]. Only three of the four sites were found to be occupied by nucleotides, when analyzing the crystal structure of a heterotrimeric AMPK complex [75]. In addition, one site appears to be non-exchangeable for ADP or ATP due to a tight binding of AMP, resulting in two sites, which competitively bind AMP, ADP or ATP. These sites are most probably the sites via which cellular energy status is sensed by AMPK. The binding of AMP to the γ -subunit are suggested to promote activation of AMPK through three independent mechanisms; promotion of Thr172 phosphorylation, inhibition of Thr172 dephosphorylation and allosteric activation of AMPK already phosphorylated on Thr172 [76-78]. In addition to AMP, ADP was recently also demonstrated to activate AMPK. ADP does not however seem to activate AMPK allosterically [79, 80].

Except for the activation of AMPK by LKB1, Ca²⁺/Calmodulin dependent protein kinase (CaMKK), especially the β isoform, has been suggested to be an upstream kinase of AMPK in several cell types [81-83], and it stimulates AMPK activity in response to increases in intracellular Ca²⁺. TAK1 is also a suggested upstream kinase of AMPK, but less studied [84].

The regulation and role of AMPK in adipocytes

The activation of AMPK in adipocytes has been described to occur in response to a variety of signals, including pharmacological agents such as the AMP mimetic 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR) [85], as well as catecholamines, for example during fasting and exercise [86-88]. The detailed mechanism underlying AMPK activation in adipocytes, as well as its functional role, is not as clear as in liver and muscle, in which the regulation and role of AMPK has been extensively studied. Whilst AMPK seems to inhibit both lipolysis and lipogenesis, its role in the regulation of glucose uptake is more unclear.

Reports suggest both an increase and decrease in glucose uptake in response to AMPK activation, depending on stimuli and model used [88-91].

ACC is a key enzyme in the lipogenic pathway. It synthesizes malonyl-CoA from acetyl-CoA and was one of the first targets identified for AMPK [92]. Malonyl-CoA is an important substrate of fatty acid synthesis. Treating adipocytes with AMPK activators such as AICAR and phenformin, which both increase intracellular levels of AMP, or expressing a constitutively active form of AMPK, increased the phosphorylation of ACC in adipocytes [68, 77, 91]. The phosphorylation of ACC at Ser79 by AMPK inhibits ACC activity, resulting in a decreased lipogenesis [93]. Activation of AMPK by AICAR in adipocytes is also suggested to lead to the upregulation of peroxisome proliferator-activated receptor (PPAR) α , β and γ , as well as PPAR γ co-activator-1 α (PGC1 α) [94].

Catecholamines probably activate AMPK as a result of increased lipolysis and subsequent changes in the AMP/ATP ratio. The lipolytic pathway is important in order to provide fatty acids and glycerol to peripheral tissues. Activating AMPK in adipose tissue using AICAR has been shown to decrease β -adrenergic induced lipolysis and this was confirmed using a constitutively active and a dominant negative version of AMPK [68, 77, 93]. Some contradictory results in 3T3-L1 adipocytes do however exist [95]. The negative regulation of lipolysis by AMPK was shown to be via an inhibitory phosphorylation of HSL on Ser565 [68, 96]. This phosphorylation is thought to prevent phosphorylation of Ser563 by PKA, and inhibits HSL activity. In addition, ATGL, another important lipolytic enzyme, was suggested to be a substrate of AMPK in brown adipose tissue [97]. Future studies will have to address a possible regulation of ATGL by AMPK also in WAT.

It may seem strange that AMPK activation should restrict the release of energy substrate to other tissues. However, a high rate of lipolysis is energy demanding from an adipocyte perspective. The FAs released can be re-esterified into acyl-CoA, which is an ATP-consuming process. In addition, free FAs are known as mitochondrial uncouplers and their accumulation in adipocytes, could therefore lead to a reduced production of ATP [98]. The activation of AMPK by catecholamines probably functions as a feed back mechanism limiting the energy loss in the cell, which is associated with lipolysis [99, 100]. Supporting the idea of an inhibition of lipolysis is the phenotype of the general catalytic α 1-subunit AMPK knock out mice [68]. The phenotype includes a reduced adipocyte size and an increased basal and stimulated lipolysis.

AMPK-related kinases

The AMPK-related kinases share sequence homology in their catalytic domain with AMPK (Figure 2) [5], and 11 (exception MELK) out of the 12 related kinases identified are all activated by LKB1 via phosphorylation of a conserved threonine residue in their T-loop [50]. Although much is today known about AMPK and its regulation and role in several cellular processes, the roles of the related kinases are just emerging.

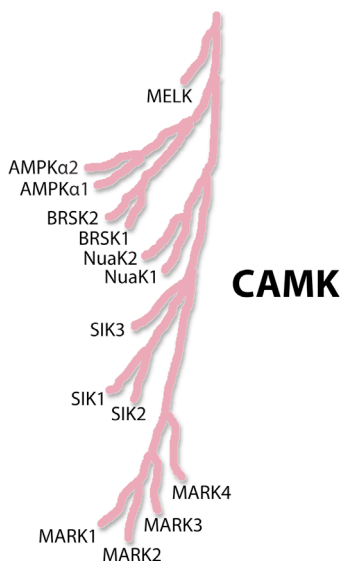


Figure 2 Dendrogram of AMPK and its related kinases

Selected part of the human kinome dendrogram, where AMPK and its related kinases are found in the group of CAMK. The human kinome classifies kinases into groups, families and sub-families primarily based on their catalytic domain. Adapted from *Manning et al 2002* [5].

The AMPK-related kinase family comprises BRSK1-2 (Brain-specific kinase), NUAK1-2 (NUAK1 and NUAK2 are also known as ARK5 and SNARK, respectively), SIK1-3 (Salt-inducible kinase), MARK1-4

(microtubule affinity-regulating kinase) and MELK (maternal embryonic leucine zipper kinase). In contrast to AMPK, AMP and Ca^{2+} do not appear to activate AMPK-related kinases [50, 52], although there is still some controversy in this area [18, 101, 102]. The AMPK-related kinases also share the feature of a ubiquitin-associated (UBA) domain, shown to be important for the phosphorylation by LKB1 [103]. Interestingly, for AMPKα1 and α2, the UBA domain was lost during evolution. This further distinguishes the AMPK-related kinases from AMPK.

NUAK1 has been suggested to be involved in cell detachment [104] and senescence [105], via phosphorylation of myosin phosphatase target 1 and large cell tumor homolog 1, respectively. NUAK2 has been shown to have anti-apoptotic properties [106] and a metabolic role has also been suggested studying NUAK2-deficient mice. Furthermore, NUAK2 has been suggested to mediate

contraction-stimulated glucose uptake in muscle [107] and very recently NUAK1 was also suggested to be involved in glucose homeostasis [14]. Deletion of the *Nuak1* gene in mouse muscle resulted in improved glucose homeostasis during high fat diet (HFD) conditions and the mice were suggested to be resistant to HFD-induced insulin resistance. NUAK1 was reported to be phosphorylated by PKB, leading to an increased activity of the kinase [102]. However, a recent report using a specific mutant of the PKB site (Ser600Ala) did not confirm this increase in activity [105].

One of the first roles described for MARKs was to phosphorylate microtubule-associated proteins and thereby cause their dissociation from microtubules [108]. MARK1 is suggested to be involved in the regulation of synaptic plasticity [109] and studies using mouse lines deficient in MARK2 indicates diverse roles, including a role in glucose homeostasis and energy metabolism [11]. MARK3-deficient mice displayed similar phenotypes [13]. Both MARK2 and MARK3 knock-out mice, however also suggests a role for these kinases in development and growth. Disruption of the *Mark4* gene in mice resulted in resistance to diet-induced obesity [12]. In contrast to MARK2 and MARK3 null mice, disruption of the *Mark4* gene did not result in growth retardation. TAO1 (thousand and one amino acids) [110] and glycogen synthase kinase 3 β (GSK3 β) [111] have both been suggested to be upstream kinases of MARKs, with activating and inhibiting phosphorylation, respectively. In addition, the MARKs are also reported to be regulated by atypical protein kinase C (aPKC) [112]. The MARK isoforms, along with SIK1 and 3, interact with 14-3-3 proteins [113, 114] and the binding of 14-3-3 to MARKs involves several residues, but do not seem to have an effect on its catalytic activity [115]. The regulation by aPKC was suggested to enhance the binding of 14-3-3s and to re-locate MARKs to the cytosol from the plasma membrane [112, 116]. In COS-7 cells, MARK2 and MARK4 were found to phosphorylate serine residues of CREB regulated transcription co-activator (CRTC) [117], which is also a suggested substrate of AMPK and the SIKs, as further described in the section “Salt-inducible kinases (SIKs)”. MARK2 also seems to phosphorylate CRTC2 at Ser275 in β -cells [118].

BRSK1 and 2 are suggested to be involved in regulating the polarity of neuronal cells [119, 120], which was confirmed in studies using mice lacking both BRSK1 and BRSK2 [121]. With regards to regulation there are conflicting reports of a role for PKA as an upstream kinase of BRSK2 [101, 122], whereas the regulation of SIK1 by PKA is more established. The regulation and role of the SIKs are further introduced in the section “Salt-inducible kinases (SIK)”.

MELK is expressed at high levels in both embryonic and neural stem cells. Suggested roles for MELK include enhanced gene transcription via interaction with zinc-finger proteins and inhibition of spliceosome assembly [123, 124]. MELK is in contrast to the other AMPK related kinases activated via autophosphorylation, and is thus not dependent on LKB1.

In summary the AMPK-related kinases are involved in diverse cellular processes and have been confirmed to take part in at least some of the tumor suppressor effects of LKB1. However, as AMPK, the related kinases also take part in the regulation of metabolism, perhaps not by sensing energy as AMPK, but rather via other mechanisms, which are not yet completely understood. The main activator of AMPK-related kinases seems to be LKB1, although other upstream kinases may also alter the function, mostly by regulating other aspects than the intrinsic kinase activity.

Salt inducible kinases (SIK)

SIK1, -2 (also known as QIK) and -3 (also known as QSK) constitute one sub-family of the AMPK related kinases and have recently received interest due to their relation to AMPK and suggested role in the regulation of hepatic glucose output [21, 125].

Expression and structure

SIK1 was first identified from the adrenal gland of rats fed a high salt diet, hence the name salt-inducible kinase [126, 127]. The two additional isoforms, SIK2 and SIK3 were identified by sequence homology to SIK1 in data base searches [20]. The SIK1 gene is located on chromosome 21q.22.3, whilst genes for SIK2 and SIK3 are located close to each other on chromosome 11q23.1 and 11q23.3 respectively. Expression profiles of the SIK isoforms reveal an abundant expression of SIK1 in the adrenal gland [128, 129], high expression of SIK2 in adipose tissue [18, 20], and a ubiquitous expression of SIK3. These patterns of expression do not exclude the possibility that SIK isoforms may be important in tissues where they display a relatively low level of expression. Potential regulation and roles of SIK isoforms have in fact been described in several tissues and cell models. All three isoforms differ in molecular mass and have a large variability in their C-terminal regulatory regions. As all AMPK-related kinases, the SIKs contain a highly conserved kinase domain in their N-terminal region, harboring the T-loop, which is important for kinase activity, followed by a UBA domain (Figure 3). The UBA domain is suggested to be important for maintaining the structure of the SIKs and for their phosphorylation by LKB1 [103]. Although the SIK isoforms are not found to bind ubiquitin chains in *in vitro* assays, a recent study suggests that the UBA domain is of importance for the localization of SIK1 in ubiquitin clusters [130]. Also, SIK1 was found to bind ubiquitinated proteins in cells and this interaction was dependent on the UBA domain, but not the activity of the

kinase. Mutating residues in the UBA domain resulted in a loss of punctate localization of SIK1 within the nucleus [103]. The C-terminal region of SIK1 houses a putative nuclear localization signal (NLS), spanning the amino acid sequence 567–612 [128, 131]. The same characteristics of a NLS cannot be found in the sequence of SIK2 or -3. The reported domains and suggested regulation via phosphorylation described below are presented in Figure 3.

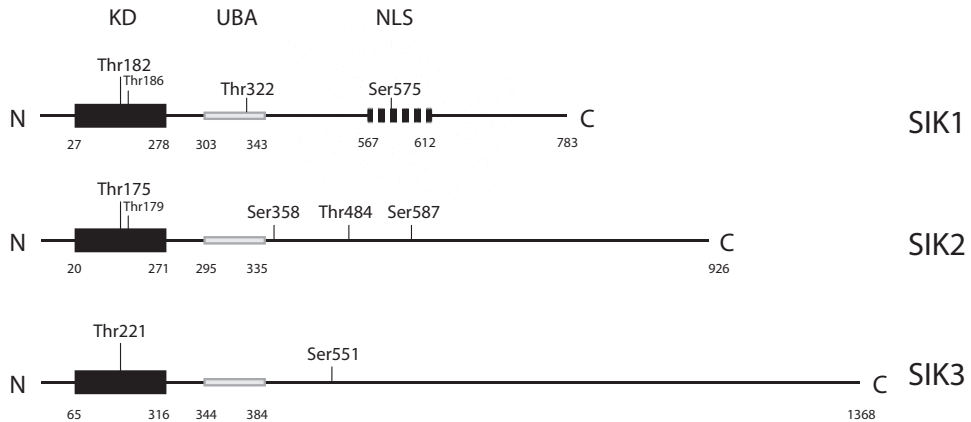


Figure 3 Structure of the SIK isoforms

The highly conserved N-terminally located kinase domain (KD) is vital for the activity of the salt-inducible kinase (SIK) isoforms. The activation loop of the kinase domain harbors an activating threonine (Thr) phosphorylation (human SIK1 – Thr182, SIK2 –Thr175, SIK3-Thr221), which is carried out by LKB1. The kinase domain is followed by a ubiquitin associated domain (UBA), common for the AMPK-related kinases. The UBA domain is important for structure and for LKB1 phosphorylation of the activation loop. In the C-terminal part of SIK1 there is a suggested nuclear localization signal (NLS), which also harbors a phosphorylation site suggested to take part in nucleo-cytoplasmic shuttling; serine (Ser) 575 (corresponding to mouse Ser577). Additional suggested phosphorylation sites of the SIK isoforms are indicated.

Regulation

The regulation of SIK2 and SIK3 gene transcription is not well characterized, but the expression of SIK1 gene transcription has been suggested to be induced in response to high salt intake [126], TGF β signaling [130, 132] and activation of the transcription factor CREB [129]. One study suggests that mRNA and protein levels of SIK2 decrease in B16 melanoma cells in response to UV-B irradiation [16] and another that SIK3 mRNA levels in liver increases with high fat feeding

[15]. Increased expression of SIK2 has interestingly been reported during adipogenesis [20].

As for almost all the AMPK related kinases, the SIK isoforms are dependent on their phosphorylation in the T-loop by LKB1 for their catalytic activity [50]. This was demonstrated in LKB1-deficient cells, and by means of mutating the T-loop phosphorylations site into a non-phosphorylatable residue, which renders these kinases completely inactive. The phosphorylation of the T-loop on Thr182 (SIK1), Thr175 (SIK2) and Thr221 (SIK3), is the most well studied regulation of the SIKs and except for LKB1 (Figure 4), no other upstream kinase have so far been shown to regulate this site.

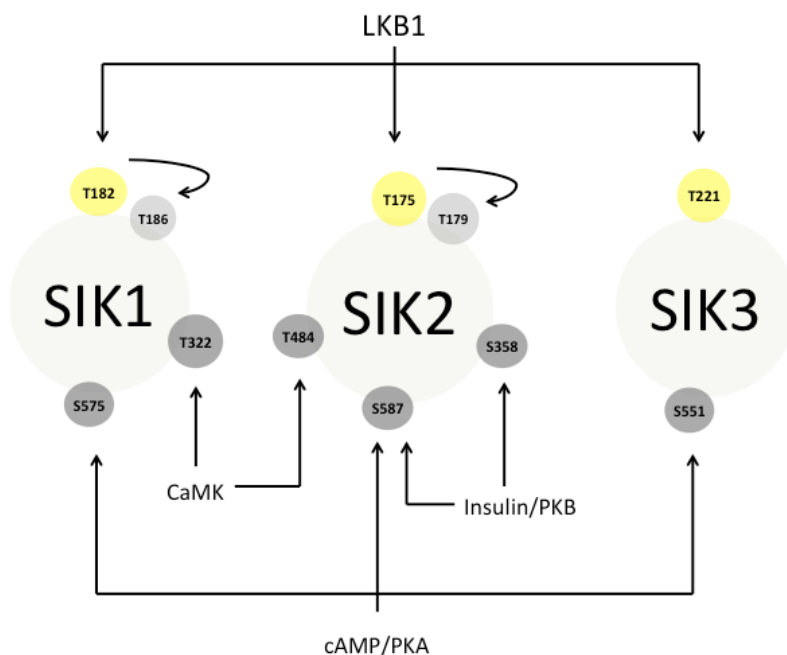


Figure 4 Overview of the suggested regulation of SIK isoforms

LKB1 is required for the activity of the salt-inducible kinase (SIK) isoforms, phosphorylating a threonine (Thr) residue in their activation loop. Also, an autophosphorylation site of SIK1 and SIK2 is suggested in a +4 position of the activating Thr residue. Protein kinase A (PKA) is suggested to phosphorylate all isoforms on serine (Ser) residues (Ser575, Ser587, Ser551) in a relatively conserved region, which in the case of SIK1 contains a putative nuclear localization signal (NLS). Phosphorylation sites regulated by insulin/Protein kinase B (PKB) are suggested for SIK2 in liver (Ser358) and brown adipose tissue (Ser587). In common for SIK1 and SIK2 is a suggested phosphorylation by Ca^{2+} /Calmoduline Kinase (CaMK) isoforms in response to sodium (Thr322 of SIK1) and ischemia (Thr484 of SIK2), respectively.

AICAR, an AMP mimetic, and nutrient deprivation, which both cause an increased AMP/ATP ratio in the cell, were suggested to increase the activity of SIK2 in 3T3-L1 adipocytes [18], but the mechanisms, including a potential effect on T-loop phosphorylation, for this were not presented. There is also one report suggesting that autophosphorylation of SIK1 and -2 within their T-loop creates a priming site for phosphorylation of a neighboring residue by GSK3 β , which results in increased stability and thereby activity of the proteins [133]. Using a GSK3 β inhibitor, the degradation of SIK1/2 proteins in COS-7 cells and in differentiating C2C12 myocytes was induced [133, 134]. Ser577 of SIK1 (human Ser575) and the corresponding site Ser587 of SIK2 is suggested to be phosphorylated in response to increased levels of cAMP and this site is also conserved in SIK3 (Ser551) [128, 135]. Ser577 is located in the NLS of SIK1 and is therefore suggested to be responsible for the nuclear export of SIK1 seen in repose to cAMP-elevation [128, 131, 135, 136]. Ser587 of SIK2 was recently also suggested to be phosphorylated in response to insulin in brown adipose tissue [137], suggesting a tissue specific regulation of SIK2 phosphorylation. cAMP-induced phosphorylation of SIK1 and 2 is described to result in decreased cellular function – conclusions based mainly on the constitutive nuclear localization and ability of cAMP-resistant mutants, for example Ser577Ala SIK1, to stimulate gene expression [128, 135, 136]. Isoproterenol and angiotensin II treatment of epithelial cells [138] and cells derived from renal proximal tubules [139], respectively, are however suggested to result in increased SIK1 kinase activity. Insulin has been suggested to activate SIK2 through phosphorylation of Ser358 by PKB, in hepatocytes [21]. Also, Thr322 of SIK1 and Thr484 of SIK2 are suggested to be phosphorylated in response to sodium in epithelial cells and ischemia in neuronal cells respectively. This regulation seems to involve Ca²⁺/Calmoduline kinase (CaMK), which is activated in response to increased intracellular calcium [17, 140]. A summary of the suggested regulation of SIK by phosphorylation is presented in Figure 4.

Substrates and interacting proteins

A consensus motif for the phosphorylation by SIKs has been presented; (LXBS/TXSXXXL) [19], where L represents Leucin, sometimes presented as any hydrophobic residue and B represents a basic residue. The consensus sequence was generated based on peptide assays, measuring the phosphotransferase ability of SIK2 toward selected peptide substrates. A summary of suggested substrates of SIK1-3 are presented in Figure 5.

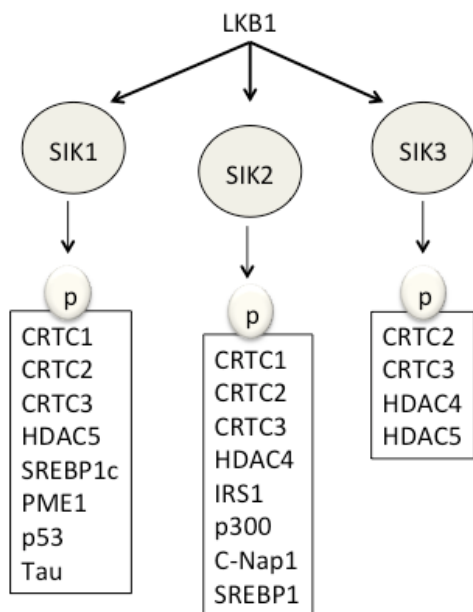


Figure 5 Suggested substrates of the SIK isoforms

Summary of salt-inducible kinase (SIK) substrates as suggested by the literature. The list also includes substrates that have not necessarily been established as *in vivo* targets, such as proteins shown to be phosphorylated *in vitro*.

Suggested substrates are as follows; CREB regulated transcriptional co-activator (CRTC), histone deacetylase (HDAC), sterolregulatory element-binding protein (SREBP), protein phosphatase methylesterase-1 (PME1), tumour suppressor protein p53, insulin receptor substrate-1 (IRS1), histone acetyltransferase p300, the centrosomal protein C-Nap1 and Tau, a protein that stabilizes microtubules.

Common suggested substrates for the SIKs are proteins within the class II histone deacetylase (HDAC) and CRTC families. CRTCs are phosphorylated by SIKs in the basal state, inducing the interaction of CRTCs with 14-3-3 proteins [19, 141] and a subsequent trapping in the cytosol (Figure 6). The activities of SIK1 and -2 towards CRTCs have been suggested to be inhibited by cAMP signaling and PKA phosphorylation, resulting in a dephosphorylation and nuclear import of CRTCs [19, 125]. Several phosphatases, especially calcineurin in response to calcium signaling, have also been shown to be important for the dephosphorylation and nuclear import of CRTCs. In the nucleus, CRTCs participate in the regulation of CREB target gene transcription. The class II HDACs are also known to be sequestered in the cytoplasm, via binding to 14-3-3 proteins, when phosphorylated by SIK isoforms [134, 142-146]. The class II HDACs are commonly known as

transcriptional repressors and have described roles in regulating genes involved in glucose metabolism both in liver and adipocytes [147, 148]. A role in adipogenesis has also been suggested [149]. The phosphorylation of these proteins by SIKs is suggested to be involved in transcriptional regulation in β -cells [19], skeletal muscle [142], melanocytes [16], adrenal cells [150], liver [21, 125] and brown adipose tissue [137]. The best characterized phosphorylation site of CRTCs suggested to be phosphorylated by SIKs is that of Ser171, but attention has also recently been brought to additional sites, Ser275 and Ser307 [117, 118]. Class II HDACs are phosphorylated on Ser246, Ser259 and Ser155 in HDAC4, 5 and -7 respectively. As is also true for the sites phosphorylated by SIKs in CRTCs, these residues lie in a sequence that confers with the SIK consensus motif described [142, 143, 151, 152].

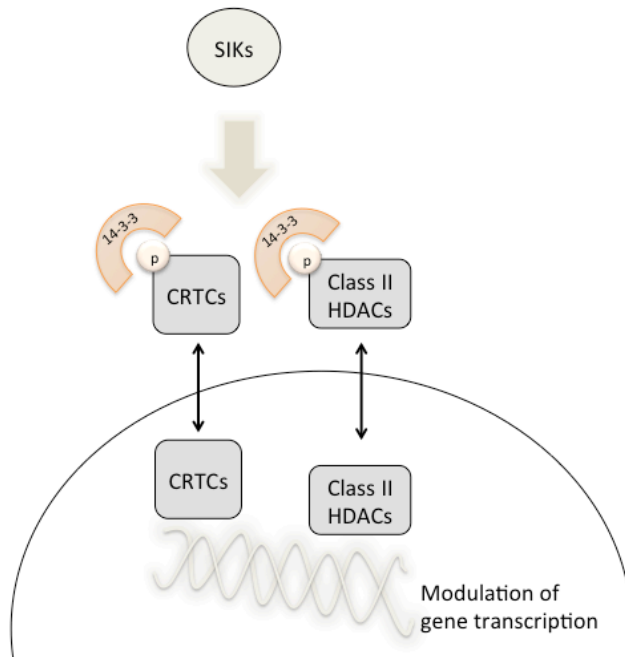


Figure 6 Modification of gene regulation via CRTCs and Class II HDACs

The most well studied biological role of SIKs involves gene regulation via CREB regulated transcription co-activator (CRTCs) and class II histone deacetylases (HDACs). When phosphorylated CRTCs and class II HDACs interact with 14-3-3 proteins and are located in the cytoplasm, thereby unable to act on gene transcription.

Another protein involved in acetylation and a suggested substrate of SIK2 in liver, is the histone acetyltransferase (HAT) p300 [153]. Ser89 phosphorylation of p300 by SIK2 was demonstrated in the liver and shown to be important in the regulation

of lipogenic genes via the transcription factor carbohydrate-responsive element-binding protein (ChREBP). In the liver, ChREB acts in synergy with sterol regulatory element-binding protein 1c (SREBP1c) to fully activate fatty acid synthesis. SIK1 has also been suggested to phosphorylate SREBP1c [154] and in 3T3-L1 adipocytes SIK2 is suggested to reduce the nuclear translocation of endogenous SREBP1 [18].

In one of the first reports on SIK2, which for example described the abundant expression of SIK2 in adipose tissue, it was also suggested that SIK2 phosphorylates Ser794 of insulin receptor substrate-1 (IRS1) [20]. The same site was by other investigators suggested to be phosphorylated by AMPK [155], but more details of this regulation and its physiological relevance remains to be established.

More recently, additional substrates have been reported for SIKs, especially for SIK1, the most studied isoform. These substrates play a role in a wide range of cellular processes. SIK1 was suggested to phosphorylate Tau [156], a protein associated with Alzheimer's disease when hyperphosphorylated, p53, in response to cell-detachment and anoikis [157], and protein phosphatase methylesterase-1 (PME1), a methylase suggested to inhibit protein phosphatase 2A (PP2A) activity towards the Na^+/K^+ -ATPase [140].

14-3-3 proteins are molecular scaffolds that interact with proteins mainly at sites of threonine and serine phosphorylation, which often leads to their accumulation in the cytosol [158]. Interestingly, AMPK-related kinases, including the SIKs, often introduce 14-3-3 binding sites into their substrates, but also bind to 14-3-3 proteins themselves [114, 115]. This binding has been shown to result in increased activity and/or a re-localization of the kinases [112, 114-116]. In SIK1 and SIK3, the T-loop phosphorylation (Thr182 and Thr221 respectively) was found to mediate 14-3-3 binding, and the interaction had an impact on the activity and localization of these kinases [114]. In the case of MARK2 and MARK3, other AMPK-related kinases known to interact with 14-3-3 proteins, the binding was primarily mediated by phosphorylations occurring outside of the kinase domain, and only affected the sub-cellular localization.

Furthermore, SIK1 was reported to interact with Smad7 and Smurf2, which was linked to the degradation of TGF β type I receptor/ALK5 (ALK5) and negative regulation of TGF β signaling [130, 132]. SIK1 was also found to co-immunoprecipitate with Na^+/K^+ -ATPase in opossum kidney (OK) cells [140] and in MLE-12 cells [138]. SIK2 or SIK3 was not found in this complex. The interaction with Na^+/K^+ -ATPase was suggested to be via a larger complex, which also includes PP2A and PME1. The nature of this interaction has not been further investigated. PP2A was also found to co-purify with SIK2 when analyzing potential interacting partners using a mass spectrometry approach. Additional confirmation of this interaction was not presented [114]. An interaction was however confirmed in immunoprecipitates of the SIK isoforms from COS-7 cells

overexpressing SIK1-3 and all subunits of PP2A [117]. In addition, SIK1 was found to be part of a complex including PP2A in OK cells, as described above [140]. Detailed mechanisms underlying SIK-PP2A interaction, for example whether it is a direct or indirect interaction, is however lacking. The interaction found in COS-7 cells is suggested not to be via the kinase domain, as expressing only this part did not result in a co-immunoprecipitation of PP2A [117]. Using Okadaic acid (OA) in low concentrations have earlier been shown to inhibit PP2A and this treatment was suggested to stabilize the phosphorylation of SIK1 at Ser577 as measured by downstream effects of this phosphorylation in COS-7 cells. OA is however, when used in higher concentrations also an inhibitor of PP1. Regulation of AMPK by AMP (or ADP) is mediated via binding of the nucleotide to specific motifs in the regulatory γ -subunit. In contrast to AMPK, no such AMP- or ADP binding subunits have been identified for the SIKs [114]. Based on this, it does not seem likely that SIK isoforms can directly sense or respond to changes in cellular AMP/ATP levels.

Biological role

Highly diverse functions have been described for the SIK isoforms (Figure 7). The tumor suppressor LKB1 being an upstream kinase of the SIKs, as well as the relation of SIKs to the energy sensor AMPK, has prompted investigations of a potential role of these kinases both in cancer and in the regulation of metabolism. With regards to metabolic roles of SIK isoforms, the literature is mainly focused on their involvement in negative regulation of the hepatic gluconeogenic program [21, 125], which was demonstrated to be enhanced in LKB1-deficient mice [159]. Their suggested role in relation to cellular processes involved in cancer include cell polarity [160], mitosis [161, 162] and p53 dependent anoikis (apoptosis induced by cell detachment) [157]. In addition, increased levels of SIK2 was associated with poor survival in ovarian cancers and silencing of SIK2 seem to sensitize ovarian cancers to paclitaxel [161]. SIK3 has also been described as a tumor-associated antigen in ovarian cancer [163]. However, decreased expression of SIK1 was found to correlate with the development of distal metastases in breast cancer [157].

The most well characterized role of the SIK isoforms is their ability to regulate gene expression through their action on various transcriptional regulators, like CRTCs and class II HDACs (Figure 6).

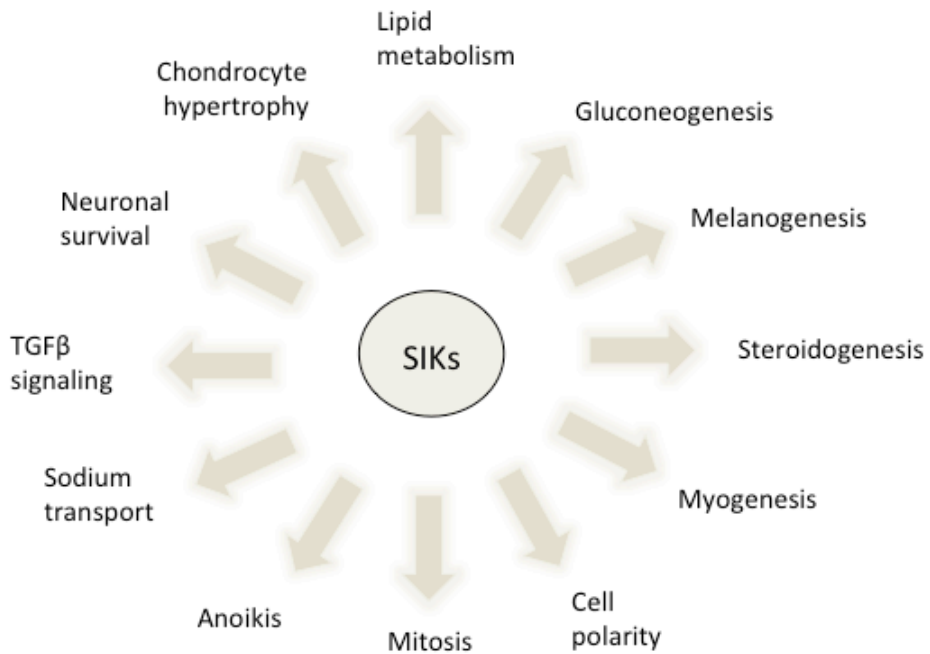


Figure 7 Biological roles suggested for the SIK isoforms

The salt-inducible kinases (SIKs) are suggested to be involved in diverse cellular functions as indicated by arrows.

CREB-mediated transcription of target genes, which contain specific cAMP-responsive elements (CRE) in their promoters, is activated in response to cAMP and calcium via phosphorylation of CREB on Ser133. Full CREB activity also requires co-activators, such as CRTC, which via binding to CREB increases its activity [164, 165]. The regulation of CREB target genes by the SIK isoforms is suggested to mainly be by phosphorylating CRTCs. In this manner, SIK1 is suggested to inhibit steroidogenic gene expression in the adrenal gland [128, 129, 135, 150, 166, 167] and gluconeogenesis in the liver [117, 125], both effects resulting in the repression of CREB activity. In SIK2-deficient mouse models, the resistance to oxidative stresses after brain ischemia [17] and enhanced melanogenesis [16], was mediated through modulation of CREB activity. Further more modulation of CREB activity by SIK2 was also observed in the regulation of gluconeogenic genes in the liver [21] and thermogenic genes in brown adipocytes [137] by SIK2. An increased nuclear accumulation of CRTC2 was observed in hepatocytes of SIK3-deficient mice, implying a role also for SIK3 in the regulation of CREB activity [15]. Moreover, neuronal silencing of a drosophila version of the

SIK isoforms (dSIK2) resulted in flies more resistant to starvation via increased CRTC2/CREB signaling [168].

The regulation of class II HDACs by SIK isoforms has suggested to play a role in energy metabolism and the myogenic program. An SIK isoform in drosophila (dSIK3) was recently demonstrated to be involved in the regulation of energy balance in the fat body, shown to be mediated through the action of dSIK3 on HDAC4 [143]. The role of HDAC4 suggested in drosophila involved deacetylation of the transcription factor FOXO and in fact an increased gene transcription, in contrast to the well-known role of class II HDACs as transcriptional repressors. Interestingly, the transcription of the drosophila version of the lipolytic enzyme ATGL, was identified as one of the FOXO target genes that were regulated downstream of the SIK/HDAC pathway in the fly. SIK2 was also suggested to be involved in the regulation of HDAC4 in mouse hepatocytes [143]. The regulation of another class II HDAC, HDAC5, by SIK1 was reported in muscle [142], resulting in the activation of MEF2C transcriptional activity. Viral expression of SIK1 was found to rescue muscle dystrophy in a transgenic mouse model expressing dominant negative CREB (ACREB). Recently, mice deficient in SIK3 were described [15]. The SIK3-deficient mouse model suggests a role for SIK3 in glucose- and lipid metabolism in the liver, as well as a role in cholesterol and bile acid homeostasis, involving Vitamin A metabolism. The lean phenotype seen was suggested to involve accumulation of HDAC5 in the nucleus of hepatocytes. Further more, studies using SIK3-deficient mice also report a role in skeletal formation, with an important role in chondrocyte hypertrophy [169]. The regulation of class II HDACs by SIK3 in cartilage was found of importance, since HDAC4 accumulated in the nucleus of SIK3-deficient chondrocytes. Suggestively, this lead to the suppression of MEF2C, an important facilitator in chondrocyte hypertrophy.

As mentioned above, a recent report also suggests that the HAT p300 is phosphorylated and thereby inhibited by SIK2 [153]. Regulation of p300 by SIK2 suppressed the ChREBP-dependent gene transcription of lipogenic genes, and silencing of SIK2 in the liver resulted in the development of liver steatosis. Over expressing SIK2 in liver protected from liver damage induced by HFD. Knock-down of SIK1 in liver also resulted in steatosis, with increased expression of lipogenic genes like *Fas*. The suggested SIK-dependent regulation of hepatic steatosis in this study was via SREBP1c phosphorylation [154].

The SIK2-deficient mice presented [16, 17] only displayed a slight obese phenotype when fed low-fat diets and detailed investigation of a potential metabolic phenotype in these mice is needed to clarify whether SIK2, like SIK3, takes part in of glucose- and lipid metabolism. Furthermore, SIK1-deficient mice do not seem to display any significant metabolic phenotype [160].

SIK3-deficient mice display decreased levels of *Scd1* and *Fas* in the liver. When silencing SIK2 expression in the liver using hepatic vein injection of adenovirus

encoding SIK2 siRNA, the effect seen was the opposite, with increased expression of genes involved in *de novo* lipogenesis [153]. In line with this data, there is one report describing that in coxsackievirus and adenovirus receptor-expressing (CAR) 3T3-L1 adipocytes, silencing of SIK2 resulted in increased mRNA expression of *Fas*, *Acc2* and *Scd1* [18]. Furthermore, in adipose tissue from *db/db* mice, the expression and activity of SIK2 was found to be increased [20]. However, in the liver of *ob/ob* mice and mice fed a high-fat diet, the activity of SIK2 was found to be reduced [153]. Collectively, both induction and negative regulation of lipogenic gene expression by SIKs has been presented so far and future investigations will have to clarify if this is partly due to tissue and isoform-specific differences.

SIK1 is the most studied of the SIK isoforms and in addition to what is described above it has been implicated in a number of different contexts. For example, additional roles have been suggested. SIK1 was reported to be important for the regulation of cardiomyogenesis [170] and this involved the cell cycle inhibitor p57. SIK1 is also involved in regulation of Na⁺/K⁺-ATPase activity by sodium [140], and in TGFβ signalling [130, 132]. Increased intracellular levels of sodium in epithelial cells increased the activity of SIK1, resulting in an increased Na⁺/K⁺-ATPase activity. The increased activity of Na⁺/K⁺-ATPase does not involve a direct effect of SIK1 on the channel, but is rather induced via the contributions of a larger complex including PME1 and PP2A, as described previously [140]. Furthermore, hormonal regulation of Na⁺/K⁺-ATPase activity, for example in response to isoproterenol and angiotensin II, requires the incorporation of new subunits into this complex [138, 139, 171]. Interestingly, cytoskeletal proteins such as kinesins display consensus sites for SIK1, and it is therefore possible that SIK1 not only takes part in the activation of Na⁺/K⁺-ATPase when at the membrane, but may also regulate the trafficking of subunit-containing vesicles, as isoproterenol increased the activity of SIK1 in MLE-12 cells [138].

TGFβ signaling is involved in the regulation of several cellular processes and has been shown to induce the expression of SIK1 [130]. SIK1 forms a complex with Smad7 and Smurf2, an ubiquitin ligase, leading to the down-regulation of ALK5 via lysosomal and proteasomal processes [130, 132]. In this way SIK1 functions as a negative feedback mechanism in the regulation of established TGFβ genes, involving for example the cyostatic program including cell cycle inhibitors like p21.

Collectively, the biological roles of the SIK isoforms are highly diverse, reflecting several of the suggested functions of LKB1, both in cancer and metabolism, but also other functions not previously linked to LKB1. The most well characterized role so far is the regulation of gene transcription by SIKs, through the phosphorylation of important transcriptional regulators. However, more recently suggested substrates also imply a role for SIK isoforms in acute regulation of cellular functions.

AIM

The overall aim of the work presented in this thesis has been to investigate the regulation of LKB1 signaling pathways in adipocytes, with a focus on the AMPK-related kinase SIK2. Establishing the regulation of LKB1 signaling pathways in adipocytes will provide important clues of the potential roles of this signaling in adipocyte function. The more specific aims were:

- To identify upstream kinases of AMPK, SIK2 and SIK3 in adipocytes
- To investigate the regulation of AMPK, SIK2 and SIK3 by extracellular signals in adipocytes
- To identify interacting partners and molecular targets of SIK2 in adipocytes
- To investigate the biological role of SIK2 in adipocytes

METHODOLOGY

Adipocyte models

The adipocyte models used in this thesis were 3T3-L1 adipocytes, isolated primary rat adipocytes, isolated human adipocytes and adipose tissue and isolated adipocytes from LKB1^{Hypo} (fl/fl) mice.

3T3-L1 is a pre-adipocyte cell line, which under the right conditions can differentiate into adipocytes [172]. The cell line is polyclonal and was derived from murine embryonic tissue. 3T3-L1 adipocytes have been widely used for hypothesis testing, enabling larger experiments with more conditions and the sparing of animals. An obvious advantage of the 3T3-L1 model is that these cells can, even in their adipocyte form, be kept in culture for extended periods of time, whereas primary adipocytes are difficult to culture more than up to 24h. For this reason, 3T3-L1 adipocytes were employed for siRNA silencing experiments, which in general require the cells to be cultured for 48-72h in order for proteins expression to be silenced. Adipocytes are difficult to transfect however, electroporation proved to be an efficient way of administrating siRNA into these cells.

Primary rat adipocytes were isolated from the epididymal fat pad of Sprague Dawley rats. Primary rat adipocytes represent a more physiologically relevant model as they are much more similar to human adipocytes than the cultured 3T3-L1 adipocytes are, storing lipids in one large lipid droplet. 3T3-L1 adipocytes on the other hand accumulate lipids in several smaller droplets. Primary rat adipocytes are well suited for adenoviral transduction and used for overexpression of SIK2. The effect of SIK2 overexpression on adipocyte function was then monitored. 3T3-L1 adipocytes on the other hand, are poorly transduced with adenoviruses since they lack the adenovirus receptor. We also isolated human adipocytes from subcutaneous as well as visceral depots of subjects undergoing gastric bypass surgery, using a similar procedure as described for primary rat adipocytes [173].

In Paper I, we used adipose tissue and isolated adipocytes from epididymal fat pads of a mouse model with decreased expression of LKB1 (LKB1^{Hypo}, fl/fl), donated by Prof. Alan Ashworth (Institute of Cancer Research, UK) [174].

HEK293 cells

In addition to adipocytes, human embryonic kidney (HEK293) cells were used for overexpression of SIK2 and SIK3 and their site-specific phospho mutants. HEK293 cells are easy to culture and are easily transfected, compared to adipose cells. As described in detail in Paper II, we also employed HEK293 cells to generate cell lines with inducible, stable expression of wild type and Ser358Ala SIK2. These cells were used for immunopurification of SIK2 and potential binding partners, as well as for activity measurements, evaluating the importance of Ser358 for SIK2 activity. Stable transfection is advantageous because it enables clones with a similar, and in our case relatively low, level of expression of the exogenous protein. A low expression level ensures a favorable ratio between bait and interacting proteins in co-purification experiments. It also reduces the risk of the exogenously expressed protein not being regulated in the same manner as the endogenous one.

In vitro kinase activity measurements

Determining protein kinase activity and how it is regulated by extracellular as well as intracellular signals is an important step in elucidating signal transduction pathways. *In vitro* kinase activity measurements of immunoisolated kinases using radiolabelled ATP, which gives high specificity and sensitivity, were used in all papers included in this thesis. Most protein kinases can be assayed using peptide substrates, as a short sequence is often sufficient for substrate recognition. In Paper II, we evaluated several peptide substrates for the activity measurement of SIK2. Peptide substrates are often selected based on protein sequences surrounding phosphorylation sites on known target proteins, as were the substrates evaluated in Paper II. Apart from a good peptide substrate, the kinase of interest also needs to be immunoisolated with high specificity. We tested the specificity of our antibodies by analyzing immunoprecipitates for the presence of kinases with similar substrate specificity, such as other SIK isoforms. The amount of antibody for the immunoprecipitation, as well as the amount of lysate used in the assay, was optimized in order to ensure linearity and that the amount of antibody was not limiting. In addition, to confirm activity measurements towards peptide substrates, we also used truncated and full-length proteins (HDAC5 1-550 in Paper II, and CRTC2 in Paper III) as substrates for SIK2 and SIK3, and the phosphorylation of these proteins was detected by autoradiography. An advantage of using full-length proteins is that potential docking sites that lie further away from the

phosphorylated residue are available, and a real interaction between the substrate and the kinase is possible.

Localization studies

In Paper II and Paper III, immunocytochemistry was used to study the localization of SIK2 and SIK3, respectively. Primary adipocytes are challenging in many ways, and one thing making them harder to use in an immunocytochemistry approach is the specific architecture of the cell, with a large lipid droplet and a very thin surrounding cytoplasm. We therefore took use of HEK293 cells which overexpressed tagged (HA- or GFP) SIK2 or SIK3. Confocal microscopy was used to analyze the localization. One of the main advantages of using confocal microscopy, over conventional (wide-field) microscopy, is that it minimizes background fluorescence, thereby allowing the imaging of a specific focal plane. Since HEK293 cells are not physiological relevant for SIK2 and were mainly used as an expression system, we also decided to use a fractionation approach to study the localization of SIK2 in primary adipocytes. Our studies using immunocytochemistry did not suggest a nuclear localization and therefore a crude fractionation protocol was used, centrifuging at 60 000 x g to separate the cytosol from a particulate fraction. We also hypothesized that SIK2 might locate to specific compartments via interaction with other proteins and therefore chose a low ionic strength buffer to optimize the conditions for these potential interactions.

Identification of interacting proteins

To identify proteins that could associate with SIK2 and participate in its regulation, or themselves be regulated, we performed large-scale immunoisolation of SIK2 from HEK293 cells stably expressing the kinase. Co-purifying proteins were then identified by tryptic digest and detection of the peptides by liquid chromatography (LC) – tandem - mass spectrometry (MS/MS) [175]. In the unpublished data presented, LC was used to separate the peptides that were to be further analyzed by MS. Electrospray ionization (ESI) was used to charge and deliver the ions to the MS right after the LC. MS analysis is performed to determine the m/z (mass/charge) values of the ions present. Using the orbitrap classic, the five most intense ions were selected for MS/MS analysis. Collision-induced dissociation (CID) was used to fragment the selected ion/ions and generate a daughter ion spectrum. The spectra created are submitted to a database search system, Mascot. The MS data was analyzed as a series of patterns and

compared to a similar set of data derived from a theoretical digest available in the relevant database. The use of LC-MS/MS and CID is an accepted standard for protein identification. To confirm the interactions observed in HEK293 cells, we overexpressed HA-SIK2- wild type (wt) and GFP in adipocytes and blotted for co-immunoprecipitating proteins. We also studied a potential regulation by cAMP expressing the SIK2-Ser358Ala mutant.

In Paper II and III, we also took use of pull-downs with the interacting protein, in this case 14-3-3, as bait for SIK2 or SIK3 as well as a Far western approach (also known as overlay), in which recombinant 14-3-3 was allowed to bind to SIK2 or SIK3 on the nitrocellulose membrane. One advantage of this technique is that it gives information about whether 14-3-3 can bind directly to the protein in question – an issue that can be difficult to address when employing different types of co-purification approaches.

Analysis of specific phosphorylation sites

Protein phosphorylation is a common and important modification as it can lead to alterations in the activity, localization, stability and interactions of a protein. It is helpful to determine the exact amino acid (Serine, Threonine or Tyrosine) on which a protein is modified, in order to understand the upstream mechanism behind the phosphorylation and its potential effects. In Paper II and Paper III we used phosphopeptide mapping to identify potential phosphorylation sites of SIK2 and SIK3, respectively, induced by increased levels of cAMP in cells. Individual phosphorylation sites are often only partially phosphorylated and therefore available at low yield in the protease digest, making the study of phosphorylations by mass spectrometry challenging. Especially two approaches have been developed to overcome this challenge: phosphopeptide mapping, a pre-enrichment of the phosphopeptides prior to MS analysis and a selective detection in MS based on the unique fragmentation behavior of phosphopeptides. Serine, threonine and tyrosine phosphorylation is selectively detected using a precursor ion scan of m/z - 79 (PO_3^-), which is followed by a determination of the peptide sequence containing the phosphorylation using MS/MS. In Paper II, the activation loop was present in a sequence not cleaved by trypsin and thereby too large for analysis. This sequence contains the activating Thr175 site of SIK2, which was therefore not identified in our study. This is one limitation of this method. However, digestion with additional/other proteases might give more details regarding this specific sequence.

In the papers included in this thesis we also employed consensus antibodies recognizing sites phosphorylated by PKA and PKB. This is helpful when phospho-specific antibodies are lacking or the identity of the phosphorylation sites is yet

unknown, but their use can also be limited, due to overlap in their consensus sites, as shown in Paper II and III. The significance of PKA for the phosphorylations we identified was confirmed using several inhibitors. In addition, with the information from the phosphopeptide mapping and site-directed mutagenesis studies that followed, we produced a phosphospecific antibody targeting phospho-Ser358 of SIK2 (Paper II). This enabled confirmation of the phosphorylation of endogenous SIK2 on this site, and can in the future also be used as a tool in read-outs of SIK2 function coupled to this phosphorylation. In addition, by using this phosphospecific antibody we were able to confirm the phosphorylation of this site in human adipocytes (unpublished data presented in “Results and Discussion”). Furthermore, several additional established phosphospecific antibodies were used as controls throughout the studies.

Lipid and glucose metabolism in adipocytes

In Paper I, D-[6-³H]-glucose incorporation into adipocyte triglycerides was analyzed as a measure of lipogenesis. The assay is based on the use of a toluol-based scintillation liquid to measure only the glucose that has been incorporated into lipids. To analyse the effect of SIK2 on the expression of genes involved in adipocyte metabolism, we also used qPCR and western blotting, in particular to monitor the expression of key enzymes in lipogenesis (presented as unpublished data). In addition, glucose uptake assay (measuring the uptake of ¹⁴C-glucose) and lipolysis assay (measuring the glycerol released) were used for evaluating a role of SIK2 in adipocyte function (Paper IV and unpublished data).

MAIN FINDINGS

Paper I

- AMPK α 1 activity is decreased in adipocytes and adipose tissue from mice with reduced expression of LKB (LKB1^{Hypo}) compared to wild type
- Activation of AMPK by phenformin is reduced in LKB1^{Hypo} mice
- Basal lipogenesis is significantly reduced in adipocytes from LKB1^{Hypo} mice
- AMPK is activated by CaMKK in response to increased intracellular Ca²⁺ in adipocytes
- The physiological Ca²⁺ increasing stimulus thyroid hormone (T₃) activated AMPK
- SIK2 and SIK3 activity is reduced in adipose tissue of LKB1^{Hypo} mice

Paper II

- HDAC5tide is an efficient substrate for measurements of SIK2 activity *in vitro*
- Increased cAMP levels using a β -adrenergic receptor agonist in adipocytes results in a PKA-dependent phosphorylation of SIK2, with no effect on its intrinsic kinase activity
- Increased levels of AMP (AICAR, phenformin), Ca²⁺ (ionomycin) or stimulation with insulin does not change the activity of SIK2 in adipocytes
- The adaptor protein 14-3-3 binds SIK2 in response to cAMP elevation in adipocytes
- Phosphopeptide mapping of SIK2 identified several sites induced by increased levels of cAMP (Ser343, Ser358, Thr484, Ser587)
- Site-directed mutagenesis revealed that Ser358 is important for the PKA-dependent phosphorylation and was important for 14-3-3 binding to SIK2 in adipocytes

- Endogenous SIK2 is phosphorylated at Ser358 in primary adipocytes
- SIK2 undergoes a phosphorylation dependent re-localization from a particulate fraction to the cytosol in response to increased cAMP levels in adipocytes

Paper III

- SIK3 is phosphorylated, with increased binding to 14-3-3 proteins, in response to the β -adrenergic receptor agonist CL316,243 in adipocytes
- Stimulation of adipocytes with CL316,243 results in a decrease in kinase activity of endogenous SIK3
- Several residues (Thr469, Ser551, Ser674) were identified to be involved in the regulation of SIK3 in response to increased cAMP, using phosphopeptide mapping
- Thr469 appears to have the most dramatic, Ser551 moderate and Ser674 modest effect on SIK3 phosphorylation and 14-3-3 binding
- 14-3-3 binding sites generated in response to cAMP elevation are most likely directly phosphorylated by PKA

Paper IV

- Phosphorylation of CRTC2 and class II HDACs is reduced following LKB1 silencing in adipocytes
- Endogenous SIK2 regulates the phosphorylation of CRTC2, CRTC3 and HDAC4 in adipocytes
- SIK2 kinase activity is important for the phosphorylation of CRTC2, CRTC3 and HDAC4
- CRTC2, CRTC3 and HDAC4 are dephosphorylated in response to increased levels of cAMP in adipocytes
- Dephosphorylation of HDAC4 at Ser246 is reduced in adipocytes expressing the cAMP insensitive SIK2-Ser358Ala mutant
- CRTC2, CRTC3 and HDAC4 interact with SIK2 in adipocytes

- The interaction of CRTC2 or CRTC3 with SIK2 is decreased in response to increased levels of cAMP in adipocytes
- SIK2-Ser358Ala interaction with CRTC2 or CRTC3 is not reduced to the same degree as SIK2-wt in response to increased levels of cAMP
- Basal glucose uptake seems to be increased in adipocytes expressing SIK2-wt

Unpublished data

- Ser358 of SIK2 is phosphorylated in response to increased levels of cAMP in human adipocytes
- PP2A regulatory subunit (reg)A, PP2A regB and PP2A catalytic subunit (cat) along with protein phosphatase 1B (PP1B alternative name PP2C isoform β) were identified using LC-MS/MS in an anti-FLAG-SIK2 purification from HEK293 cells
- PP2A regA and PP2A cat interact with SIK2 in HA-immunoprecipitation of SIK2 from adipocytes.
- The interaction between the PP2A regA subunit and SIK2 is reduced in response to cAMP elevation and does not change when expressing SIK2-Ser358Ala
- *Glut4*, *Scd1* and *Acc* mRNA levels are decreased in adipocytes silenced for SIK2
- SCD1 and ACC protein levels are decreased in adipocytes silenced for SIK2

RESULTS & DISCUSSION

Regulation of AMPK, SIK2 and SIK3 in adipocytes (Paper I, II, III and unpublished data)

Regulation by energy stress and the master upstream kinase LKB1

In adipocytes, AMPK has been demonstrated to be activated in response to a variety of agents. However, the upstream molecular mechanisms, including the identity of upstream (T-loop) kinases, underlying this activation have so far not been addressed. Therefore, the aim of Paper I was to study the role of the two most well-known upstream kinases of AMPK, LKB1 and CaMKK in the regulation of AMPK in adipocytes. We took advantage of a mouse model with reduced expression and activity of LKB1 (LKB1^{Hypo}) to demonstrate the importance of this upstream kinase for the activity of AMPK and the AMPK related kinases SIK2 and SIK3 in adipocytes. As shown in Paper I, phosphorylation of AMPK at the critical Thr172 site, and thereby the activity of AMPK, was significantly reduced in both adipose tissue and isolated adipocytes from LKB1^{Hypo} mice, compared to wild type mice. Also, the activities of SIK2 and SIK3 were found to be significantly reduced, however not to the same extent as AMPK. The importance of LKB1 for the activity of AMPK and most of its related kinases has previously been demonstrated in several cell systems [49-51]. Our findings confirm that LKB1 is an upstream kinase of AMPK, SIK2 and SIK3 also in adipocytes. The residual activity of these kinases detected in LKB1^{Hypo} mice, could be due to additional upstream kinases and/or the low amounts of LKB1 still present in LKB1^{Hypo} mice [81, 83]. It has indeed been reported that in skeletal muscle of the same mice, AMPK could still be activated by phenformin, but not in mice with a muscle-specific, complete ablation of LKB1 in these tissues [174]. A mouse model in which LKB1 is specifically deleted in adipose tissue would perhaps represent a more robust model to study the requirement of LKB1 in the regulation of AMPK and its related kinases. However, such a model has so far not been presented.

One important conclusion from Paper II is that AICAR and phenformin, known AMPK activators via changes in AMP/ATP ratio do not seem to affect the activity of SIK2 in 3T3-L1 adipocytes, in contrast to an earlier study suggesting AICAR and nutrient deprivation to activate SIK2 in CAR 3T3-L1 adipocytes. Our data is however in line with other studies, which also do not support an impact of AMP

on the kinase activity of SIK2 [50, 114]. So far there is no subunit binding AMP identified for SIKs, as is the case for AMPK. We also investigated the effect of glucose starvation on SIK2 activity in 3T3-L1 adipocytes (data not shown). Preliminary data from SIK2 activity measurements do not support earlier findings, suggesting increased activity in response to nutrient deprivation [18], but will have to be further evaluated.

Regulation by Ca^{2+} and CaMKK

We also addressed the role of CaMKK β and Ca^{2+} in the regulation of AMPK and SIK isoforms, and found that the physiological Ca^{2+} inducer, thyroid hormone (T_3) was able to increase the phosphorylation and activity of AMPK in adipocytes, and that this activation was dependent on CaMKK β (Paper I). Thyroid hormones are known to regulate metabolism and function of many tissues, including adipocytes, where suggested roles include both increased lipolysis and lipogenesis. The functional role of the Ca^{2+} -dependent activation of AMPK that we describe, remains to be addressed.

In Paper 1, we also found that the CaMKK inhibitor STO-609 did not influence the activity of SIK2 or SIK3, arguing against a role for CaMKK in the regulation of these kinases. In agreement with this finding, increased intracellular Ca^{2+} did not influence the activity of SIK2 in 3T3-L1 adipocytes (Paper II). Collectively, our data suggest that calcium is not important for the regulation of SIK2 kinase activity in adipocytes. Interestingly, CaMKI/IV was reported to phosphorylate SIK2 at Thr484 in response to increased intracellular levels of Ca^{2+} in cortical neurons, leading to its degradation [17]. Future studies could address whether this site is phosphorylated in response to raises in Ca^{2+} in adipocytes. A possible regulation of SIK3 by CaMKK was not investigated in detail.

Regulation of SIK2 and SIK3 by cAMP-elevating agents and insulin

An important step in elucidating the biological role of the AMPK-related kinases SIK2 and SIK3 in adipocyte is to characterize in which manner these kinases are regulated by cellular stimuli, for example hormones. LKB1 appears to be constitutively active, and in contrast to AMPK, the T-loop phosphorylation and activity of which is induced by changes in AMP/ATP and Ca^{2+} levels, we did not find that SIK2 responded to any of these signals. However, it is possible that AMPK-related kinases are subject to regulation that does not directly involve the kinase domain or changes in T-loop phosphorylation and activity. In Paper II and III, we investigated such a potential regulation of SIK2 and SIK3 in response to cAMP-elevating hormones and insulin - two critical signals controlling adipocyte

function. In our studies, agents that elevate cAMP were found to regulate both SIK2 (Paper II) and SIK3 (Paper III) at many different levels, including their phosphorylation by PKA on multiple sites, but also with regards to their interaction with other proteins (described in “Identification of interacting partners and molecular targets of SIK2 & SIK3”). The regulation of SIK isoforms by cAMP/PKA, especially SIK1, has earlier been demonstrated in Y1 cells and 3T3-L1 fibroblasts. Since the suggested PKA site in the regulation of SIK1 by cAMP-elevating agents, Ser577 (human Ser575), is located within a putative NLS, it is not surprising that a phosphorylation induces the nuclear export of SIK1. Neither SIK2 nor SIK3 display the same properties in their amino acid sequence, although the region containing the suggested PKA site (Ser575 in SIK1) is conserved in these isoforms (Ser587 in SIK2, Ser551 in SIK3). We performed a phosphopeptide mapping in order to directly identify all cAMP-induced phosphorylation sites of SIK2 and SIK3 in HEK293 cells and in adipocytes (SIK2, Paper II). This approach is unique in the sense that it provides a more complete picture of which phosphorylations that occur, as compared to for example western blotting using phosphospecific antibodies. The mapping did not only reveal sites induced in response to cAMP, but also provided interesting information about the basal phosphorylation status of the proteins. SIK3, as compared to SIK2, is a much larger protein and was found to be heavily phosphorylated in its far C-terminal parts in the basal state. These phosphorylation sites do not seem to be due to autophosphorylation, since the kinase inactive version (HA-SIK3-D206A) displayed a phosphorylation pattern that was almost identical to the wild type protein (Paper III). It is interesting that these phosphorylations were found especially in the regions not conserved between the SIK isoforms. The role of these phosphorylation sites in SIK3 function remains to be further assessed. Furthermore, in contrast to SIK2, in which a specific phosphorylation site (Ser358) was identified as the major site contributing to the overall PKA-induced phosphorylation, the phosphorylation of SIK3, as detected by PKA consensus antibodies, was only fully reversed when the majority of the phosphorylated sites were mutated to Ala. One site did however seem to influence the PKA phosphorylation more than others, namely Thr469 (Paper III). Mapping all sites phosphorylated in SIK2 and SIK3 in response to cAMP represents a more direct strategy compared to earlier studies, which only employed sequence motif analysis followed by site-directed mutagenesis. Our studies identified Ser358 as the most important site for the regulation of SIK2 by cAMP in adipocytes. This is in contrast to findings in other tissues and cell types, in which Ser587 was suggested to be important for the effect of cAMP/PKA on SIK2 function. In the liver, Ser358 was reported to be phosphorylated in response to insulin. In contrast to that study, insulin was not found to regulate SIK2 or SIK3 in our experiments, as judged by monitoring changes in activity (Paper II), phosphorylation using consensus

antibodies targeting PKB phosphorylation sites (Paper II and Paper III) and a phosphospecific antibody against SIK2 pSer358 (Paper II).

In order to investigate whether the cAMP-mediated regulation that we described for SIK2 in Paper II is also relevant in humans, we have now extended our studies to isolated human adipocytes from both subcutaneous and visceral depots. As shown in Figure 8, using anti-SIK2 pSer358 antibodies, we have demonstrated that, like in rodent cells, Ser358 is phosphorylated in response to β -adrenergic agonists in human adipocytes. The finding that the regulation of SIK2, by phosphorylation of Ser358, is conserved in human adipocytes points to an important role for this regulation. Future studies will have to address if SIK2 is phosphorylated on additional sites, in response to cAMP-elevating agents or other agents, in human adipocytes. It would also be of interest to investigate if SIK2 is differentially expressed and/or regulated in obese or insulin resistant subjects.

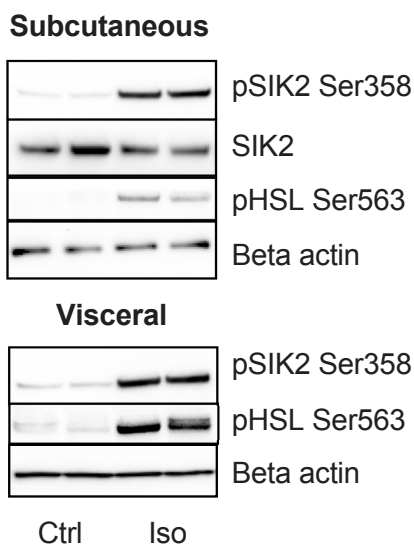


Figure 8 Ser358 phosphorylation of endogenous SIK2 in human adipocytes.

Human adipocytes from subcutaneous and visceral depots were isolated and stimulated with 100 nM isoproterenol (Iso) for 30 min. Lysates from adipocytes were analyzed for pSIK2 Ser358 and total SIK2 using western blot. pHSL Ser563 and beta actin were used as controls. Representative blots from 2-3 individual experiments are shown.

The functional importance and consequences of the regulation of SIK2 and SIK3 by cAMP/PKA is still not entirely clear. Although there were no significant changes in the intrinsic kinase activity of SIK2 in response to cAMP, as was found for SIK3 in the form of an

inhibition, it is still possible that PKA phosphorylation of SIK2 inhibits its cellular function. We found that the phosphorylation of SIK2 regulates its subcellular localization (Paper II) as well as its interaction with other proteins (Paper II and Paper IV, discussed below). Based on these data, as well as suggestions in the literature that SIK1 function is inhibited by PKA phosphorylation, we hypothesize that the phosphorylation of SIK2 by PKA may restrict the access to its substrates. The result of the re-localization of SIK2 could of course also potentially lead to the opposite; increased cellular function by increased accessibility to substrates.

In conclusion, it seems that the regulation of SIK2 function by cAMP-elevating hormones in adipocytes depends on phosphorylations changing the localization of the kinase and the accessibility to other proteins, rather than direct effects on enzyme activity.

Identification of interacting partners and molecular targets of SIK2 and SIK3 (Paper II, Paper III, Paper IV and unpublished data)

cAMP-induced interaction with 14-3-3 proteins

14-3-3 proteins bind specific pSer/pThr motifs in target proteins, thereby trapping them in the cytosol. The binding can also induce conformational changes and mediate interaction to other proteins [158]. SIK1 and SIK3 have previously been shown to bind 14-3-3 proteins via their phosphorylated T-loop, enhancing their catalytic activity. It is thought that this binding to 14-3-3 proteins induces a structural change, which stabilizes SIK1 and SIK3 in an active conformation [114]. In Paper II and III, the cAMP-induced phosphorylation sites found to be of importance for the overall PKA-dependent phosphorylation were also found to mediate binding of SIK2 and SIK3 to 14-3-3. Changes in the cAMP-induced phosphorylation of SIK2 and SIK3 closely corresponded to the interaction with 14-3-3, and after mutation of the PKA sites, binding was lost. In SIK3 several sites were identified to be of importance for the binding of 14-3-3 proteins in response to cAMP (Paper III). This is in line with findings regarding the reported binding of 14-3-3 to another AMPK-related kinase, MARK3, which needed to be mutated at 17 serine and threonine residues before the 14-3-3 interaction was ablated [115]. In contrast to MARK3, SIK1 and SIK3, SIK2 did not bind 14-3-3 in unstimulated cells, and primarily one site was important for the interaction with 14-3-3 proteins in response to cAMP-elevation; Ser358 - a site that matches the most common 14-3-3 binding motif [176]. A good stoichiometry of the interaction to 14-3-3 proteins was confirmed when visualizing SIK2, SIK3 and co-precipitating proteins after immunoprecipitation and colloidal staining. All seven isoforms of 14-3-3 was found to interact with both SIK2 and SIK3, as analyzed by mass spectrometry. The interaction with 14-3-3 to Thr221 (the T-loop) of SIK3 was previously demonstrated to increase its kinase activity. Contrary to this, interaction of 14-3-3 to cAMP-induced phosphorylation sites in SIK3 was associated with a decrease in activity. As mentioned above, the activity of SIK2 was not affected by cAMP-induced 14-3-3 binding. One known effect of 14-3-3 binding is to trap proteins in the cytoplasm. However, this was not observed when investigating a potential

effect of 14-3-3 binding on the localization of SIK2 and SIK3 using immunocytochemistry of HEK293 cells expressing the two kinases (Paper II and Paper III). The binding of SIK3 to 14-3-3 via Thr221 was suggested to result in punctuate structures in the cytosol, a pattern that was not observed in our study. However, interestingly, using a fractionation approach in primary adipocytes, a re-localization from a particulate fraction to the cytosol coincided with the PKA-dependent phosphorylation and 14-3-3 binding of SIK2. It is possible that the re-localization observed is from a particulate fraction not visible by immunocytochemistry, or there might be cell specific behaviour. The analysis of the localization of endogenous SIK2 and SIK3 in resting adipocytes, as well as in response to cAMP, would be highly interesting, reflecting upon the possible artefacts using overexpression systems. Due to the characteristic architecture of primary adipocytes, immunocytochemistry of this cell type is challenging. However the analysis of endogenous SIK2 in 3T3-L1 adipocytes is ongoing. The finding that SIK2 and SIK3 interact with 14-3-3 proteins as a result of PKA phosphorylation provides a more functional read-out for the regulation of these kinases in response to cAMP-elevation.

cAMP-regulated substrates of SIK2

For a protein to be considered a genuine kinase substrate, several aspects needs to be full-filled, some of which include *in vitro* phosphorylation, a matching consensus sequence, effects when overexpressing and silencing the suggested upstream kinase, and possibly a direct interaction between substrate and kinase. In other tissues than adipocytes, CRTCs and class II HDACs have been suggested to be downstream targets of SIKs [19, 118, 125, 142, 143]. Their known regulation by cAMP, in combination with our finding that SIK2 is regulated by cAMP, made us interested to investigate if these transcriptional regulators are targets of SIK2 also in adipocytes (Paper IV). If so, we also wanted to study if the PKA-regulation found for SIK2 (more specifically, phosphorylation of the Ser358 residue) is involved in the regulation of these molecular targets by cAMP. The data presented in Paper IV, together with previous *in vitro* phosphorylation studies [19, 142, 143, 152], along with a matching consensus sequence, indeed suggests that CRTC2, CRTC3 and HDAC4 are substrates of SIK2 in adipocytes. AMPK and its related kinases are, as described earlier, dependent on LKB1 for its activity and in Paper IV we found that 3T3-L1 adipocytes with markedly decreased expression and activity of LKB1 displayed reduced phosphorylation of class II HDACs as well as Ser275 phosphorylation of CRTC2. SIK2 is abundantly expressed in adipocytes and modulating the levels of SIK2 using adenoviral expression in primary adipocytes and siRNA targeting SIK2 in 3T3-L1 adipocytes resulted in alterations in the phosphorylation of CRTC2, CRTC3 and HDAC4 (Paper IV). Using a

cAMP-insensitive mutant version of SIK2 (Ser358Ala), the regulation of SIK2 by PKA-dependent phosphorylation could be connected with some aspects of the known cAMP regulation of CRTCs and HDACs. Additional experiments are however needed to confirm this connection. In 3T3-L1 adipocytes treated with siRNA targeting SIK2, phosphorylation of HDAC4 at Ser246 and CRTC2 at Ser275 was low, and could not be further decreased by cAMP-elevation. This suggests a major role for SIK2 in the regulation of CRTC2 and HDAC4 by cAMP. A very recent report identified CRTC3 sites phosphorylated by SIK2 in macrophages [177]. Several residues (Ser62, Ser162, Ser329, Ser370) had to be substituted for Ala in order to fully abolish the phosphorylation and cytosolic trapping by SIK2. In addition CRTC2, -3 and HDAC4 were all found to interact with SIK2 in adipocytes. Interestingly, the interaction between SIK2 and CRTC2 or CRTC3, was reduced in response to increased levels of cAMP. Identifying molecular targets of SIK2 in adipocytes gives a further insight in what biological role SIK2 plays in adipocytes. If SIK3 is involved in the cAMP regulation of CRTCs and class II HDACs is still to be determined. We do not exclude that the inhibiting phosphorylations of SIK3 by PKA (Paper III) could also be involved in the regulation of these molecular targets by cAMP.

Finding out what proteins might interact with SIK2 could lead to the identification of SIK2 substrates as well as proteins that regulate SIK2 function. Since not much is known about SIK2-interacting proteins, we took use of mass spectrometry for the analysis of potential interacting partners. A low, stable expression of SIK2 in HEK293 cells was used, which maximizes the proportion of the bait that is bound to endogenous binding partners, whose expression may be limiting. Both FLAG- and GFP-tagged SIK2 was used in this study to make sure the tag did not influence the interactions. Previous work by Al-Hakim and co-workers [114], made use of a low, stable expression of TAP-tagged AMPK-related kinases in order to identify binding partners. The data from this study suggested that SIK1 and SIK3 interact with 14-3-3 proteins. Furthermore, it did not show evidence of AMPK-related kinases interacting with regulatory subunits of AMPK, or any other protein that might mediate binding to AMP or ADP. In our study we used sophisticated purification techniques available for GFP- and FLAG tagged proteins, resulting in low background with relatively few proteins present in immunoprecipitates from cells expressing the vectors alone. In addition, dithiobis succinimidyl propionate (DSP), a crosslinker that stabilizes any potential complexes, was used. As presented in Figure 9, several co-immunoprecipitating proteins appeared. When identifying the peptides from the excised bands, PP2A was identified as an interesting match. All three subunits, PP2A cat, PP2A regA, PP2A regB, were identified (Table I). The number of peptides identified, coverage of the protein sequence, in addition to the size observed of distinct bands and absence of the peptides in the control samples, makes PP2A a strong candidate for a true

interacting partner. A summary of the proteins, including all subunits of PP2A, with most abundant peptides is presented in Table I. This result confirms earlier findings from HEK293 cells using a similar system, but TAP-purification of SIK2 [114].

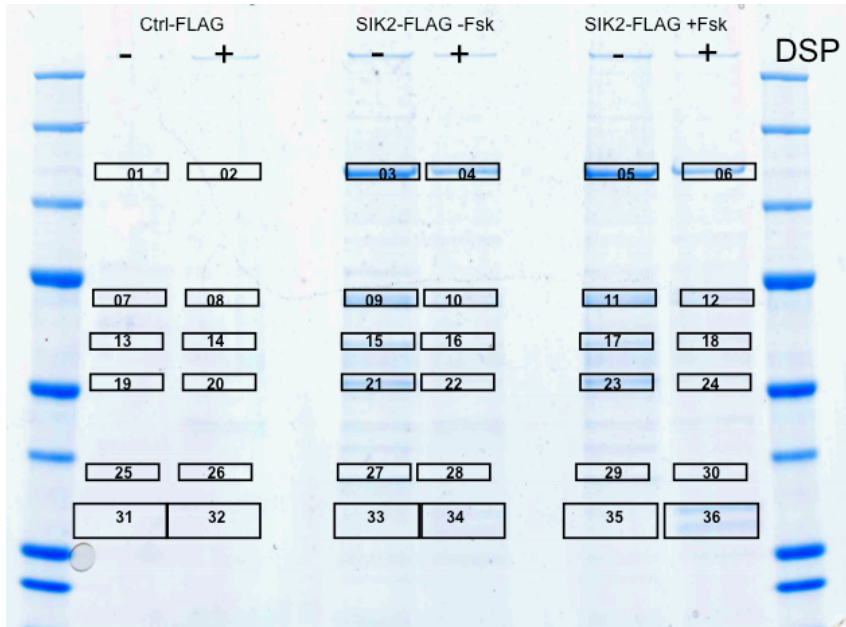


Figure 9 Anti-FLAG-purification of SIK2 and identification of interacting proteins

Anti-FLAG-purified SIK2 expressed in HEK293 cells were electrophoresed on a polyacrylamide gel and the protein bands were visualized by colloidal Coomassie Blue staining. Each of the major bands observed were excised and analyzed using liquid chromatography (LC)-tandem mass spectrometry (MS/MS).The spectra created were submitted to the database search system Mascot, were it was analyzed. Bands 1-6 represent the region of the bait (FLAG-SIK2) and bands 31-36 indicates the area for 14-3-3 proteins and has been earlier described.

Table I Summary of peptides and sequence coverage of PP2A and PP1B identified. Peptides written in red also include non-unique peptides also identified in PP2A regB alpha sequence.

Peptides	% coverage	Band # and identified sequence	Protein
17(12) 9(8) 2(2)	23 21 5	15 sp P30153 2AAA_HUMAN sp O75688 PPM1B_HUMAN sp P63151 2ABA_HUMAN	PP2A 65kDa regA alpha PP1B PP2A 55kDa regB alpha
5(4) 2(2)	6 5	16 sp P30153 2AAA_HUMAN sp O75688 PPM1B_HUMAN	PP2A 65kDa regA alpha PP1B
13(8) 13(10) 2(2)	21 18 5	17 sp O75688 PPM1B_HUMAN sp P30153 2AAA_HUMAN sp P63151 2ABA_HUMAN	PP1B PP2A 65kDa regA alpha PP2A 55kDa regB alpha
8(7) 8(7)	19 13	18 sp O75688 PPM1B_HUMAN sp P30153 2AAA_HUMAN	PP1B PP2A 65kDa regA alpha
5(5) 4(4)	12 10	21 sp P63151 2ABA_HUMAN sp Q66LE6 2ABD_HUMAN	PP2A 55kDa regB alpha PP2A 55kDa regB delta
2(2)	5	22 sp P63151 2ABA_HUMAN	PP2A 55kDa regB alpha
6(6) 3(3)	13 6	23 sp P63151 2ABA_HUMAN sp Q66LE6 2ABD_HUMAN	PP2A 55kDa regB alpha PP2A 55kDa regB delta
3(3)	6	24 sp P63151 2ABA_HUMAN	PP2A 55kDa regB alpha
8(6)	22	27 sp P67775 PP2AA_HUMAN	PP2A catalytic alpha
		28	
5 (4)	17	29 sp P62714 PP2AB_HUMAN	PP2A catalytic beta
4(4)	17	30 sp P62714 PP2AB_HUMAN	PP2A catalytic beta

We confirmed an interaction between SIK2 and the endogenous PP2A regA in primary adipocytes using adenovirally expressed HA-tagged SIK2-wt (Figure 10A). The interaction between SIK isoforms and PP2A regA was demonstrated earlier, however making use of overexpression of both proteins in COS-7 cells [117]. Interestingly, we also found that the interaction was significantly reduced in response to increased levels of cAMP, suggesting that regulation of SIK2 by cAMP may disrupt the interaction with PP2A regA. Further strengthening a regulation of the interaction via cAMP, is the preliminary finding that interaction of the cAMP insensitive mutant of SIK2, Ser358Ala-SIK2, was not changed in response to treatment with the β -adrenergic receptor agonist CL316,243 (Figure 10A).

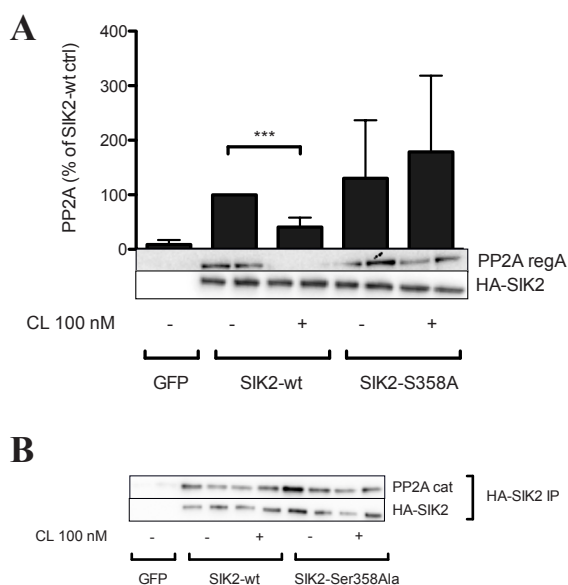


Figure 10 PP2A interacts with SIK2 in adipocytes

GFP, SIK2-wild type (wt) or – Ser358Ala (S358A) encoded by adenoviral vectors, was expressed in primary rat adipocytes (A-B). Cells were stimulated with CL 316,243 (100 nM, 30 min), HA-tagged SIK2 was immunoprecipitated (IP) from the lysates using anti HA-agarose, and co-immunoprecipitating proteins were detected by western blotting using antibodies towards (A) PP2A 65kDa regulatory subunit A (regA), (B) PP2A catalytic subunit (cat). Western blots are representative of 3-9 individual experiments (A) and 1 experiment (B). Results are presented as mean \pm s.e.m. Statistical significance of difference was defined as *** $p > 0.001$ using a paired two-tailed t test.

Preliminary data also suggest that the endogenous catalytic subunit of PP2A interacts with HA-SIK2-wt in adipocytes as shown in Figure 10B. Additional studies will have to reveal if the 55kDa regulatory subunit of PP2A (PP2A regB), identified in the mass spectrometry analysis, also interacts with SIK2 in adipocytes. Recently, an interesting connection between SIK1 and PP2A was suggested; SIK1 was found to be part of a complex with PP2A, PME1 and Na^+/K^+ -ATPase. The regulation of PP2A activity by SIK1 was suggested to occur via phosphorylation of PME1, which in a basal state demethylates PP2A. When

phosphorylated, PME1 is suggested to dissociate from this complex, resulting in increased methylation of PP2A and thereby increased activity. It is possible that the interaction between SIK2 and PP2A that we observed in adipocytes could have a similar role. This has to be further investigated and additional interacting proteins in such a complex could perhaps also appear in the data obtain from the mass spectrometry. However, in adipocytes, cell specific proteins may contribute to the interaction and/or possible regulation and future studies will include a large-scale purification of SIK2 complexes from adipocytes. One possibility is that SIK2 binds to PP2A via its interaction with CRTC2 or HDAC4, for which PP2A has been suggested to be a phosphatase in pineal cells [178] and in chondrocytes, respectively [169, 179]. In other cell systems, calcineurin was the phosphatase identified to be involved in the regulation of CRTC dephosphorylation and localization in response to calcium signalling. Calcineurin was not co-immunoprecipitated with SIK2 when expressed in COS-7 cells [117]. In addition we also identified PP1B, in the mass spectrometry data (Figure 9B). A phosphatase involved in the regulation of CRTC in adipocytes has so far not been reported. It is interesting that the decrease in PP2A interaction in response to increased cAMP levels also coincided with decreased binding of SIK2 to CRTC2 and CRTC3 (Paper IV). We do not exclude the possibility that PP2A may regulate the phosphorylation status of SIK2, as suggested earlier for SIK1 [117]. There have so far not been any extensive studies describing phosphatases in the regulation of SIK isoforms. Future studies will have to address what potential role this interaction to PP2A has and what potential adipocyte functions it may alter.

The biological role of SIK2 in adipocytes (Paper IV and unpublished data)

With its high expression in adipocytes but yet unknown regulation and role herein, one aim of this thesis was to investigate what signaling pathways might be involved in the regulation of SIK2, and thereby bring us closer to an understanding of what biological role SIK2 plays in adipocytes. With the finding that SIK2 is regulated by cAMP, we speculated that SIK2 may be involved in the regulation of lipolysis – a process which is tightly controlled by cAMP/PKA signalling. Preliminary data obtained when expressing SIK2-wt in primary adipocytes, suggest that SIK2 may inhibit basal lipolysis (Figure 11). Basal lipolysis was analyzed by measuring glycerol released into the assay medium and lipolysis from adipocytes expressing SIK2-wt was compared to cells expressing GFP control and

SIK2-Thr175Ala. The β -adrenergic receptor agonist CL316,243 was used to induce lipolysis.

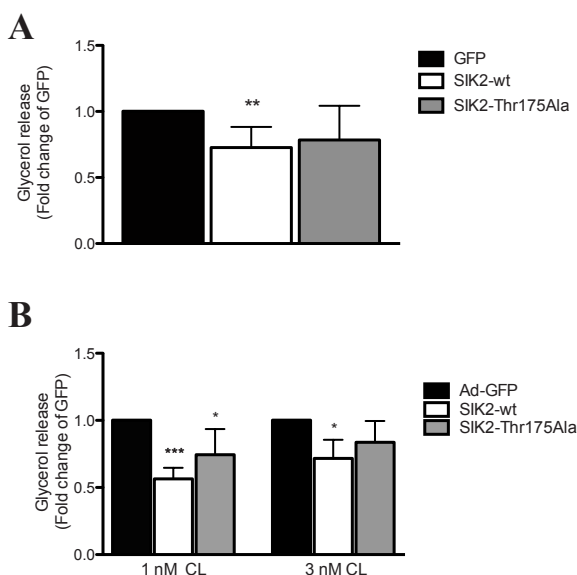


Figure 11 Adenoviral expression of wild type SIK2 decrease basal lipolysis

GFP, SIK2-wild type (wt) or -Thr175Ala (T175A) encoded by adenoviral vectors, was expressed in primary rat adipocytes over night and glycerol release was monitored before (A) and after treatment (B) with 100 nM CL316,243 (CL) for 30 min. Data presented are mean \pm s.e.m. from 5-7 individual experiments.

Identifying the class II HDAC, HDAC4, and CRTC_s as downstream targets of SIK2 suggests a potential role of SIK2 in the regulation of gene transcription in adipocytes. Both HDAC4 and CREB have been shown to be involved in the regulation of *Glut4* gene expression. GLUT4 is important for glucose uptake in adipocytes, especially the uptake induced by insulin. In Paper IV, glucose uptake was measured in primary adipocytes transduced with adenoviruses encoding SIK2-wt, SIK2-Thr175Ala or GFP. Our results suggest an increased basal glucose uptake in response to increased levels of SIK2. One could speculate that increased levels of GLUT4 might be involved in the increased glucose uptake observed. Preliminary data show decreased mRNA (Figure 12) and protein levels of GLUT4 (data not shown) in 3T3-L1 adipocytes treated with siRNA targeting SIK2, compared to scrambled-treated cells. The effect of SIK2 silencing on glucose uptake in these cells is currently being investigated.

Interestingly, in drosophila, dSIK3 was suggested to be involved in the transcription of the drosophila version of *Atgl*, a lipase important in basal lipolysis [143], via HDAC4. This, together with our preliminary data indicating that SIK2 may regulate basal lipolysis, made us hypothesize that SIK2 might regulate the expression of ATGL in adipocytes. We are currently testing this hypothesis by

means of modulation of SIK2 expression and analysis of ATGL mRNA and protein levels.

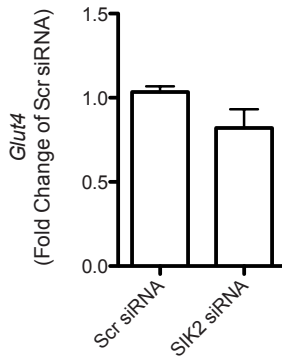


Figure 12 Silencing of SIK2 in 3T3-L1 adipocytes results in decreased levels of Glut4 mRNA

3T3-L1 adipocytes were treated with scrambled (Scr) siRNA or siRNA targeting SIK2 for 72h and *Glut4* mRNA was analysed using qPCR. The data is presented as mean of 2 individual experiments and presented as mean \pm s.e.m.

When investigating the effects of SIK2 silencing in 3T3-L1 adipocytes on a selection of proteins involved in lipid metabolism in adipocytes, we found significantly decreased protein levels of the lipogenic proteins SCD1 and ACC (Figure 13). Protein levels of FAS remained unchanged.

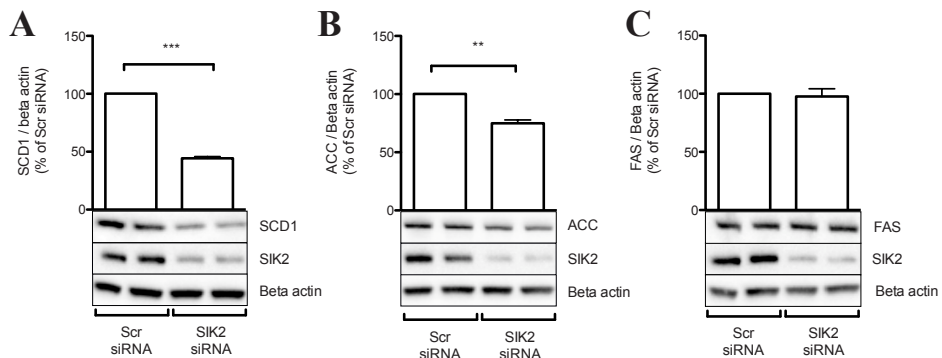


Figure 13 Silencing of SIK2 in 3T3-L1 adipocytes results in reduced protein levels of SCD1 and ACC, but not FAS

3T3-L1 adipocytes were treated with scrambled (Scr) siRNA or siRNA targeting SIK2 for 72h and protein levels of (A) SCD1 (B) ACC or (C) FAS was analyzed using western blot. The bar graph represents quantified western blot signals, corrected for total protein. The data is presented as mean of 3 individual experiments \pm s.e.m. Statistical significance of difference was defined as ** $p > 0.01$ and *** $p > 0.001$ using a paired two-tailed t test.

Preliminary data using qPCR analysis confirm these results on the mRNA level, suggesting transcriptional regulation rather than regulation of protein stability (Figure 14). These results are contradictory to a previous study suggesting that silencing of SIK2 in CAR 3T3-L1 adipocytes results in increased mRNA levels of *Fas*, *Acc2* and *Scd1*. Only FAS expression was confirmed on a protein level [18]. However, several factors could contribute to the opposing results. CAR 3T3-L1 adipocytes are often cultured in media supplemented with insulin, which differs from culturing of regular 3T3-L1 adipocytes. Notably, we found that CRTC2 and CRTC3 are differentially regulated in response to cAMP elevation in normal versus the stably transfected CAR 3T3-L1 adipocytes; whilst cAMP induces a dephosphorylation of CRTCs in 3T3L1 adipocytes and most other cell systems investigated, it resulted in increased phosphorylation of CRTCs in the CAR 3T3-L1 cell line. This provides a possible explanation to the opposing effects of SIK2 silencing on lipogenic gene expression. One could speculate that the hypomorphic mouse with reduced expression of LKB1 would provide information about how LKB1 signaling collectively affects the expression of lipogenic genes. Indeed, these mice displayed a reduction in basal lipogenesis (Paper I), which is in line with LKB1/SIK2 signaling being important to maintain lipogenic gene expression.

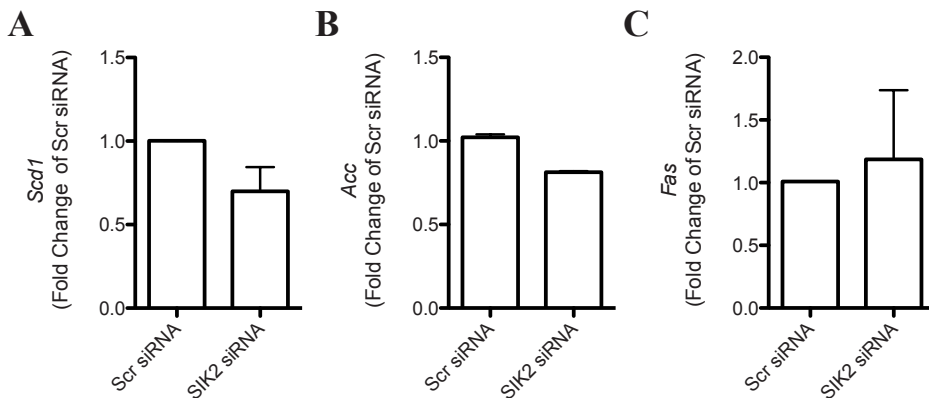


Figure 14 Reduced levels of SIK2 results in decreased levels of *Scd1* and *Acc*, but not *Fas* mRNA

3T3-L1 adipocytes were treated with scrambled (Scr) siRNA or siRNA targeting SIK2 for 72h and (A) SCD1 (B) ACC or (C) FAS mRNA was analysed using qPCR. The data is presented as mean of 2 individual experiments and presented as mean \pm s.e.m..

The biological role of SIK2 in adipocytes needs further assessment. Preliminary data in our lab suggest that SIK2 is involved in several important functions of adipocytes. Future work will have to reveal a potential role of SIK2 in obesity and type 2 diabetes. A tissue specific knock out of SIK2 in adipose tissue would be useful to determine the relevance and role of the abundant expression of SIK2 found in this tissue.

Conclusions

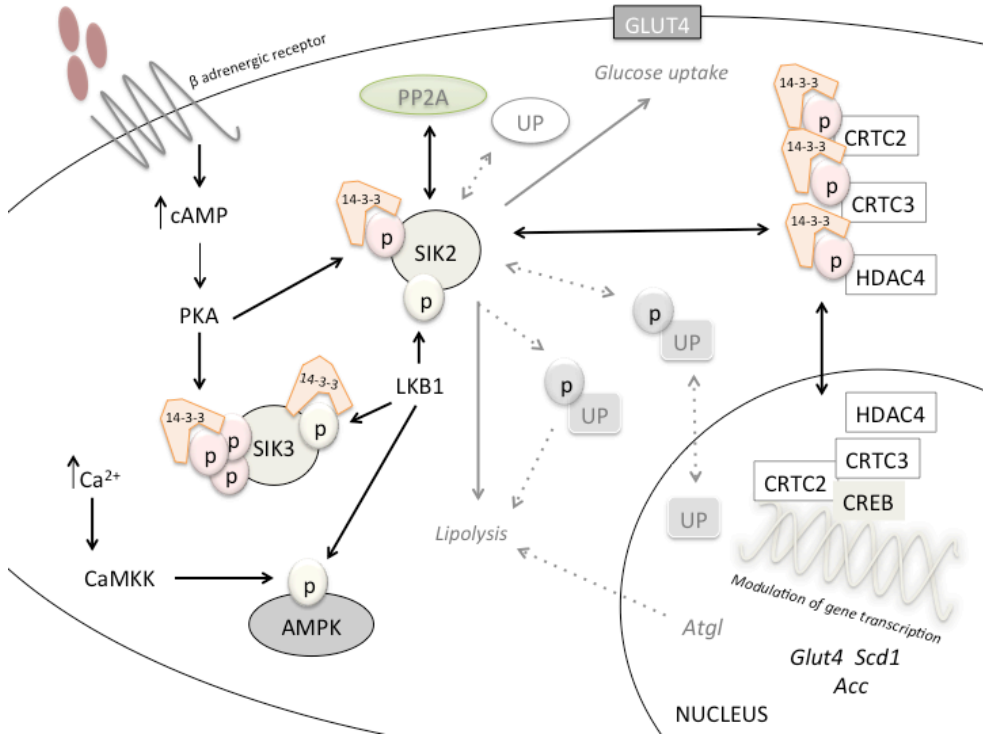


Figure 15 Summary of main findings and theories

LKB1 was confirmed as an upstream kinase of AMP-activated kinase (AMPK), salt-inducible kinase (SIK) 2 and SIK3 in adipocytes. In addition, increased intracellular calcium was found to increase AMPK activity via Ca^{2+} /Calmoduline kinase kinase (CaMKK). Increased levels of cAMP in response to β -adrenergic receptor stimulation induces a protein kinase A (PKA) dependent phosphorylation of SIK2 and SIK3, which induces binding sites for 14-3-3 proteins. The activity of SIK3 is decreased, whilst SIK2 relocates from a particulate fraction to the cytosol, as a result of this 14-3-3 binding. cAMP response element-binding protein (CREB) regulated transcription coactivator 2 (CRTC2), CRTC3 and histone deacetylase 4 (HDAC4) are suggested to be substrates of SIK2 in adipocytes, and are also regulated by cAMP in these cells. The phosphorylation sites shown to be targeted by SIK2 have previously been shown to be involved in the binding of 14-3-3 proteins. It is possible that the PKA dependent phosphorylation of SIK2 is also involved in the regulation of CRTCs and class II HDACs by cAMP. Our data also suggest a regulation of basal glucose uptake by SIK2, potentially via action on *Glut4* gene

transcription. SIK2 also seems to be involved in the regulation of stearoyl-CoA desaturase 1 (SCD1) and acetyl-CoA carboxylase (ACC) protein and mRNA levels, through a mechanism that needs to be further evaluated. We also suggest a potential role for SIK2 in the regulation of basal lipolysis; the mechanism behind this effect needs to be further assessed and could be either via acute regulation of key enzymes or through effects on gene transcription, suggestively adipocyte triglyceride lipase (*Atgl*). Protein phosphatase 2A (PP2A) was identified as an interacting protein in a mass spectrometry analysis and was confirmed to interact with SIK2 in adipocytes. The future will have to reveal the importance of this interaction and additional interacting proteins. UP stands for unknown protein, grey arrows indicate action on adipocyte function and dashed arrows indicates a suggested hypothesis.

GENERAL DISCUSSION & FUTURE PERSPECTIVES

Type 2 diabetes is in fact a complex group of diseases and several factors thus contribute to its development. The association of type 2 diabetes to obesity and thereby the role of adipose tissue in development of the disease has been recognized for quite some time. This does not only include the roles of adipose tissue as a reservoir for energy, but also its role as an endocrine organ, secreting important factors and hormones influencing glucose homeostasis in additional target tissues of insulin. Understanding the complexity of when excess adipose tissue becomes dangerous is one challenge.

This thesis attempts to address the regulation and role of important key metabolic players, protein kinases, more specifically AMPK, SIK2 and SIK3. Protein kinases are fascinating because they constitute such a large part of our genome and are crucial for the regulation of almost all cellular processes. Their broad variety in function, specificity and fine-tuned regulation, makes them good candidates for drug development. The characterization of the human kinome resulted in grouping of kinases based on similarities in their catalytic kinase domains. Structure, domains, regulation and interacting partners all bring clues into the function of a certain kinase. In this thesis we use some of these aspects to gain a greater understanding of LKB1 signaling pathways in adipocytes. We show that AMPK activity is regulated by LKB1 and also by CaMKK via thyroid hormones and increases in intracellular calcium. An adipose tissue specific knock out of LKB1 will in the future help answering some of the questions that still remains in terms of regulation of AMPK and its related kinases by LKB1 in adipocytes. There is of course also a curiosity in what phenotype this animal model might display. Future studies will also have to reveal in more detail the role of calcium signaling, and more specifically, calcium-induced activation of AMPK, in adipocytes.

In comparison to AMPK, even less is known about the regulation and role of the AMPK related kinases SIK2 and SIK3 in adipocytes. This is in spite the fact that the expression profile of SIK2 suggests an important role in these cells. The regulation of SIK2 and SIK3 by cAMP described in this thesis could be of importance for adipocyte function. In addition, the finding that CRTC2, CRTC3 and HDAC4 are substrates of SIK2 in adipocytes, implies a role of SIK2 in the regulation of gene transcription in these cells, confirming what has been observed in other tissues. We also investigated the potential functional importance of PKA-dependent phosphorylation of SIK2 for the regulation (dephosphorylation and nuclear entry) already described for CRTCs and class II HDACs by cAMP.

Further studies will have to confirm this preliminary data. Based on our investigations so far, we hypothesize that SIK isoforms take part in transcriptional regulation of genes involved in lipid and glucose metabolism in adipocytes, through their action on HDAC4, CRTC2 and CRTC3, but potentially also other transcriptional regulators. However, we do not exclude the possibility that SIK2 may also acutely regulate metabolic enzymes involved in these events. In more detail, our data suggest that SIK2 stimulates *Glut4* gene expression and glucose uptake, but our data also generated the hypothesis that SIK2 may inhibit the expression or activity of key enzymes in lipolysis, such as ATGL. ATGL has been shown to be phosphorylated by AMPK, which raises the question whether ATGL could also be a substrate of SIK2. In our attempts to identify SIK2 substrates and regulatory proteins in adipocytes, we found that PP2A interacts with SIK2 in a cAMP-dependent manner. While upstream kinases of AMPK and its related kinases are more or less well studied, very little is described about their potential regulation by protein phosphatases via dephosphorylation. The finding that SIK2 interacts with PP2A in adipocytes makes us interested in if this interaction suggests a regulation of SIK2 via dephosphorylation or vice versa. Or, is it simply as a regulator of another so far unknown protein, which then acts on PP2A, as found for the effects of SIK1 on Na⁺/K⁺-ATPase activity. So far there is no well-characterized phosphatase of the SIK isoforms, perhaps PP2A is involved in this, as SIK2 seem to interact with several subunits of PP2A in adipocytes.

The intriguing data suggesting that protein and mRNA levels of SCD1 and ACC were decreased in 3T3-L1 adipocytes treated with siRNA targeting SIK2 connects SIK2 to lipogenic processes and is in agreement with findings of increased activity and expression in adipose tissue from a diabetic mouse model. One could speculate SIK2 to be involved in the gene regulation of these key enzymes in lipid metabolism as well. To further address this, it would also be interesting to gain knowledge in the transcriptional regulation of SIK2. Also a further establishment of the regulation and biological roles found in studies from cell models, in human adipocytes, is of high interest. Future prospects also include studies correlating the activity and expression of especially SIK2, but also AMPK and additional related kinases of interest in adipose tissue from obese and healthy subjects with BMI.

SIK2 function in adipocytes is still inconclusive and this thesis is only the start of an exciting journey getting to know SIK2 and the role it may play in adipocyte function. This thesis reveals regulation important for adipocyte function and provide preliminary data connecting SIK2 to both lipid and glucose metabolism in adipocytes.

ACKNOWLEDGMENT

Life as a PhD student is not always easy. It definitely has had its ups and downs, as with everything in life I guess. A lot of blood, sweat and tears were put into this thesis. However, already before it is over, the hard times are shadowed and what I really remember is the impressive, awesome, compassionate people I met during my years as a PhD student. I remember a high coffee diet (HCD) in combinations with exciting discussions, the freedom that being PhD student brings - with worldwide conferences, not to forget the opportunities that are ahead and also me differentiating (growing up) into a scientist. I still wouldn't say I am a grownup, but as a researcher, really, who is? In the end, I am now marrying science. I am in a love-hate relationship with SIK2, he (proteins are not necessarily gender-neutral) will always have a special place in my heart and in my dreams he will rescue me from type 2 diabetes when I get old(er). Let the future tell if my dreams will come true.

There are so many persons involved in the work behind this thesis and the title it brings, my sincere thank you to all of you. One thing for sure is that without my supervisor **Olga Göransson**, I would never have had written this thesis. Thank you for this great opportunity and for trusting me in being your first PhD student. I have learnt a lot from you and I am grateful you have always (almost) understood my mumbling and that you listened to (or at least pretended to) all stupid ideas I have had. I have especially enjoyed our enthusiastic discussions about projects during my thesis writing, your ABBA singing and “hard-core-skånska” imitations. I have also had the pleasure of getting important input from my co-supervisors, both regarding specific projects and in future career choices. Thank you, **Eva Degerman**, for being a great support and for all the laughter over the years (SIK3-iller?!) and **Lars Rönstrand** for all interesting anecdotes from your research career and great company in the postgraduate research committee (FUK).

I also had the opportunity to work at the MRC protein phosphorylation unit, Dundee. **Kei Sakamoto**, thank you for the scientific input and support in my project and also thanks to **the Sakamoto group** for guiding me through MRC life. **Sabine**, thank you for all the day-trips, cappuccinos and talks during our months in Scotland. **David** and **Ivan**, thanks for great company and the runs uphill “the Law”, making me faster than ever. The **C. MacKintosh group**, thanks for taking such great care of me and for inviting me to all your events.

Also, thank you to collaborators, **Karin Stenkula**, **David Campbell** and **Nick Morrice**. Your help has been invaluable.

Everyone at BMC C11 (past and present), thank you for all the encouragement and fun over the years. Thank you, **Cecilia Holm**, for “fika-room” guidance in science, nice collaborations and advice in future career options, **Helena**, for keeping order at C11 and nice collaborations, **Martin**, my fellow crisps(chips)-maniac, every day is a crisps-day, **Birgitta**, for always caring and **Karin** for being a role model in how to be a real “tant” and for laughing at me (rather than with me). **Sara**, thanks for being you (I don’t think you actually get how much fun you are), thanks for making my bitterness seem minor in comparison to yours and for being my fellow glutard. Thanks **Mahshid**, for asking me questions, making me feel like I’ve learnt something over the years, **Lovisa** for never turning down a discussion about poo and for always pushing the limits in what’s appropriate. **Ulrika**, my mentor and “big sister” at C11, you know everything and have been a true role model both in science and in life, big hugs to you. **Toffe**, thanks for lipogenic gene-guidance and hilarious fun at fika-breaks, **Céline** for your amazing laughter and **Svante**, for being a role model in crazy-style cell culturing. **Bilal**, thank you, for being so damn clever all the time and for supporting my love for burgers. **Lena O.**, **Tina**, **Maria** and **Linda** thank you for great company and for making me eat alone, dragging everyone to the lunch room way to early. Thank you **Cecilia M.**, for Berlin recommendations and **Andreas**, the brain behind “Batman and Robin”, for the batman pimping of our office. Thank you, **Ann-Ki**, sporty spice, for always making me feel bad not running more, **Lena S.**, for input during lab meetings, **Eva O.**, for all the hours you put into my projects, for always reminding me about the dishes and for the supply of percy pig and pals, which contributed to my “avhandlingshull”. **Amélie**, thanks for the amazing smelly-cheese gratin and for all the qPCRs and **Johanna** for working your ass off as my student and for deciding to join the group as a PhD student, at least some of my science-love-spreading must have worked (or?!). Also thanks, for drosophila and consensus site discussions during my thesis writing. **Emilia**, min lilla terrier, hot twin-mama, thank you for great fun over the years, for always caring and being there if needed. **Elin**, I can’t write all your nick-names here for several reasons. Thanks for stalking me, resulting in you joining the Degis-lab. You are not only hilarious as a person, but also a great friend, always caring. Extra-special-thanks for the pimping of my office every now and then, I loved it big time. Last but not least, **Christine**, min Robin i mästerkattstövlar, Bøgen, so many memories my roomie, thank you for putting out with my bad jokes, weird discussions, my assaults (hitting) and complains over the years. Thanks for teaching me the most important Danish words there are, like “hønserv” (?). I had so much fun with you, I miss you already and I would never have made it without you.

Thanks to all **PhD students within DPLU/LUDC** making my time as a PhD student a fantastic time. All the **MDR board members**, especially my **FUK colleagues**, I had a great time and I believe we did influence things for the better. Also, thank you to the “**adult ones**” in the committee and to **Anette** and **Anna** for always bringing happy faces and answering all my confusions when I first started.

A “singing thank you“, to past and present members of the **DPLU PhD steering committee**, especially for asking me to join the group. Thanks all for hilarious planning and awesome arrangement of events. **Ola** and **Siri**, you definitely always made the meetings a challenge to run, and that’s a good thing. **Hedvig** and **Linda** thanks for bringing ideas that actually make sense, compared to, well you know who. **Cissi**, thank you for learning to understand my irony (I did like you after all). You have been an inspiration and a great support and I can see my self with you at a home for the elderly, drinking coffee, discussing science and gossiping some years from now. Looking forward to it. Thanks also for your good choice in husband. What would I have done without you?

All friends, from home-home (Kalmar), from Uni - Linköping/Lund, my London-peeps, mitt älskade Skåne-pack och annat löst folk, no one mentioned no one forgotten. Thanks for always listening to my scientific mumbo-jumbo, but most of all for always being there whenever needed, I love you guys big time. I some times say science is my life, but I know that I am wrong, you all are. You always help me remember the important things in life. I am sorry Mr Protein Kinase, even though you probably believe so and I kind of write so in this thesis, it is not you.

Except for the ones already mentioned there are some additional persons, deserving some extra loving words. **Manne** thank you for all the love, memories and support over the years and for making me appreciate the good things in life (like high-quality stuff and food). You certainly got your share of PhD student drama, words cannot describe how grateful I am you been there for me and for being part of my life. **Anders H. Olsson**, thanks for always providing a hug when needed and for finally engaging in the DPLU organization after 4 years of flirting. **Peter O.** thanks for all the inappropriate comments, no I am not pregnant, and not any of the others either. **Kalle**, you are a STAR, the best ever. You were my late night support during my time in Scotland (thanks for moving to San Diego) and you always helped out, in any way you could, especially these last months of thesis-craziness. Thanks for being such a good friend even though I forget to turn the lights of and do not eat the ends of the falukorv (Falun sausage). The words “sluta böla” and “andas” actually helped me (although annoying, of course). Big hugs to **Louise** and **Danne** for mass spec support and to **Anja** for an amazing dendrogram (4-year old style, mucho nice!). **Jonas**, du har av flera anledningar också verkligt förtjänat skiten ur ett tack, Thank you, Thank you!!

Last but not least, the most important ones in my life, **my beloved family**. I love you so much, what would I do without you. **Mom** and **dad**, I am so proud being your daughter, your support and love made me who I am today.

Mamma, jag hoppas bära med mig en bråkdel av din styrka och **Pappa**, din kreativitet har smittat av sig på mig (på gott och ont), det finns lösningar på allt, om man så ska behöva använda silvertejp! **Johan**, brollan, du har alltid varit den coola jag ville vara, tack för att du faktiskt tycker det är coolt att ha en syrra som är nörd och därmed gör mig cool (eller?!).

Farmor, önskar du varit här och fått se mig nå hela vägen fram, men jag vet att du är stolt. Tack för att jag fick komma på semester (bort från Johan) till dig och farfar. Jag hoppas bära med mig delar av din envishet igenom livet.

“The dog days are over”

Florence and the Machine

(I know, it is not really true, but that’s fine. After all - I do love science)

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