Linköping University Medical Dissertations No. 1541

**UNRAVELING MECHANISMS OF INSULIN RESISTANCE IN
TYPE 2 DIABETES IN HUMAN ADIPOCYTES**

*Role of extracellular signal regulated kinase 1/2 (ERK1/2) and
forkhead box protein 01 (FOX01)*

MEENU ROHINI RAJAN

Division of Cell Biology, Department of Clinical and Experimental Medicine
Faculty of Medicine and Health Sciences, Linköping University, Sweden

Linköping 2016

© Meenu Rohini Rajan 2016

Published articles in this thesis have been reprinted with the permission of
respective copyright holders

ISBN: 978-91-7685-677-2

ISSN: 0345-0082

Printed by LiU-Tryck, Linköping 2016

During the course of the research underlying this thesis, Meenu Rohini Rajan was
enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping
University, Sweden.

Our greatest weakness lies in giving up. The most certain way to succeed is
always to try just one more time.

Thomas A. Edison

SUPERVISOR

Professor Peter Strålfors

Department of Clinical and Experimental Medicine
Linköping University

CO-SUPERVISOR

Docent Mats Söderström

Department of Clinical and Experimental Medicine
Linköping University

FACULTY OPPONENT

Docent Olga Göransson

Department of Experimental Medical Science
Lund University

ABSTRACT

Type 2 Diabetes is characterized by hyperglycemia primarily caused due to insulin resistance in insulin responsive tissues and insufficient production of insulin by the β -cells. Insulin resistance appears to develop first in the expanding adipose tissue during caloric surplus and affects other tissues like liver and muscle by ectopic fat accumulation. In spite of significant research in field of insulin signaling, very little has been known about the mechanisms that lead to insulin resistance and T2D.

We aim for network-wide knowledge of insulin signaling in human adipocytes and to identify mechanisms that can induce insulin resistance in diabetic individuals. We have herein focused on the transcriptional control of insulin via ERK and FOXO1, and have used mathematical modelling to gain a systems-level understanding of insulin signaling network.

Through the work in this thesis, we present for the first time a dynamic comprehensive model for insulin signaling for the adipocytes, for both metabolic and transcriptional control, and that can simulate data from both normal and diabetic individuals. We described insulin regulation of ERK phosphorylation and showed that both its insulin sensitivity and maximal response to insulin was curtailed in adipocytes from diabetic individuals (Paper I). Our findings indicate that insulin regulated ERK pathway exerts control on transcription not only through phosphorylation of Elk-1 but also through phosphorylation of FOXO1 and exerts translational control via phosphorylation of ribosomal protein S6 (Paper I, II). Furthermore, we showed that insulin-induced FOXO1 phosphorylation or its insulin sensitivity was not impaired in diabetic individuals, although FOXO1 protein level was reduced by 45% in adipocytes from patients with type 2 diabetes. Comprehensive analysis of the detailed insulin signaling model showed that attenuation of the feedback from mTORC1 to IRS1-Ser³⁰⁷ explained dominant part of the insulin resistance seen in adipocytes from diabetic individuals (Paper II). More interestingly, inhibition of FOXO1 with a dominant negative construct of FOXO1, mimicked the diabetic state in the adipocytes, with the similarity extending to both insulin signaling as well as the reduced protein levels, as seen in the diabetic adipocytes. We also show that mTORC1 and FOXO1 maintain each other's expression/activity in the human adipocytes (Paper II, III). Our findings thus demonstrate that the interplay between mTORC1 and FOXO1 maintains normal insulin signaling in the human adipocytes.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, referred by their roman numerals in the text.

- I. Elin Nyman*, **Meenu R. Rajan***, Siri Fagerholm, Cecilia Brännmark, Gunnar Cedersund, and Peter Strålfors
A single mechanism can explain network-wide insulin resistance in adipocytes from obese patients with type 2 diabetes
Journal of Biological Chemistry, 2014 Nov 28, 289 (48), 33215–33230

- II. **Meenu R. Rajan**, Elin Nyman, Preben Kjølhede, Gunnar Cedersund, and Peter Strålfors
Systems-wide Experimental and Modeling Analysis of Insulin Signaling through Forkhead Box Protein O1 (FOXO1) in Human Adipocytes, Normally and in Type 2 Diabetes
Journal of Biological Chemistry, 2016 May 20, 291 (30), 15806–15819

- III. **Meenu R. Rajan**, Elin Nyman, Cecilia Brännmark and Peter Strålfors
Inhibition of FOXO1 in primary human adipocytes mimics the insulin resistant state of type 2 diabetes
Manuscript

*Authors contributed equally to the work

ABBREVIATIONS

AS160	Akt substrate of 160 kDa
ATGL	adipose triglyceride lipase
4EBP1	eukaryotic translation initiation factor 4E-binding protein 1
EGF	epidermal growth factor
Elk1	ETS domain-containing protein 1
ERK1/2	extracellular signal-regulated kinases 1/2
FFA	free fatty acid
FOXO1	forkhead box protein O1
G6Pase	glucose 6-phosphatase
GLUT4	glucose transporter type 4,
IR	insulin receptor
IRS1	insulin receptor substrate-1
MAPK	mitogen-activated protein kinases
MEK	mitogen-activated protein kinase kinase
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
p90-RSK	p90 ribosomal S6 kinase
PKD-1	phosphoinositide dependent kinase-1
PKD4	pyruvate dehydrogenase kinase 4
PEPCK	phosphoenolpyruvate carboxykinase
PI3K	phosphoinositide 3-kinase
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKB	protein kinase B
PPAR γ	peroxisome proliferator-activated receptor gamma
Rbl2	retinoblastoma-Like 2
Rheb	ras homolog enriched in brain
S6	ribosomal protein S6
S6K1	p70-S6 Kinase 1
Ser	Serine
TG	Triglyceride
Thr	threonine
TNF α	tumor necrosis factor α
TSC2	tuberous sclerosis complex 2
Tyr	tyrosine
VLDL	very low density lipoprotein

Table of Contents

- INTRODUCTION..... 1**
 - Adipose Tissue..... 3**
 - Adipocytes..... 3
 - Adipose tissue and glucose homeostasis 4
 - Insulin resistance and type 2 diabetes 5**
 - Obesity and metabolic homeostasis 5
 - Insulin resistance and adipose tissue dysfunction 7
 - Insulin signaling 13**
 - Receptor activation 14
 - Activation of IRS1 and PI3K 15
 - Regulation of insulin signaling through feedbacks via IRS1..... 16
 - Activation of PKB 17
 - Mammalian target of rapamycin 20
- RESULTS AND DISCUSSION 25**
 - ERK signaling in human adipocytes..... 27**
 - Insulin regulated phosphorylation of ERK..... 27
 - Mathematical modelling and hypothesis testing 30
 - Cross-talk between ERK and PKB-mTORC1 pathway 34
 - ERK signaling in type 2 diabetes 36
 - FOXO1 in human adipocytes 39**
 - Insulin regulation of FOXO1 activity 40
 - Regulation of FOXO1 by different post-translational modifications other than phosphorylation 41
 - FOXO1 in type 2 diabetes 43
 - Role of FOXO1 in non-adipose tissues 48
 - Role of FOXO1 in adipose tissue 50
 - Comprehensive mathematical model of insulin signaling network 55**
 - Mechanisms of insulin resistance in human adipocytes 61**
- CONCLUSIONS AND FUTURE PERSPECTIVES 67**
- POPULAR SCIENCE ABSTRACT 71**
- ACKNOWLEDGEMENTS 73**
- REFERENCES 77**

INTRODUCTION

ADIPOSE TISSUE

Adipose tissue is a highly active metabolic and endocrine organ that plays a central role in maintaining whole body energy homeostasis. It consists of a number of interacting cell types like mature adipocytes, pre-adipocytes, macrophages, fibroblasts and vascular cells which highlight the remarkable complexity of its functions.

ADIPOCYTES

Adipocytes are the primary cell type in the adipose tissue and are classically known for storage and mobilization of fatty acids to meet the nutritional requirements of the organism. Adipocyte is a large cell that can reach a diameter of upto 200 μm .

Figure 1. Adipocyte is a large cell with a unilocular lipid droplet and a thin rim of cytoplasm containing the nucleus and other organelles. The nucleus is located peripherally and the large lipid droplet pushes the nucleus against the plasma membrane.

It has a very unique structure with a large central unilocular lipid droplet that covers about 90% of the cell volume leaving a very thin film of cytoplasm

between the plasma membrane and the lipid droplet (Figure 1). Depending on the metabolic demands in the body, the adipocytes are subjected to various hormonal cues. In response to these cues, the cells take up the excess fatty acids and store as triacylglycerol in the lipid droplet allowing for the 'healthy' expansion (1) of the adipose tissue in times of over-nutrition. On the other hand, adipocytes release the free fatty acids (FFAs) from their lipid reservoir during energy deficit to provide metabolic fuel to the tissues in need. Adipocytes are also well known to secrete a variety of adipocyte derived factors called adipokines such as adiponectin and leptin that communicate with other tissues affecting the metabolism systemically [reviewed in (2)].

ADIPOSE TISSUE AND GLUCOSE HOMEOSTASIS

The plasma glucose level is tightly regulated by the two hormones - insulin and glucagon, and involves cross-talk between several organs such as pancreas, muscle, adipose tissue, liver and the brain. Plasma glucose level in healthy individuals is maintained in a narrow range of 4-7 mM despite fluctuations in energy status during the day. The anabolic hormone insulin acts as a primary sensor and regulator of glucose homeostasis in the body (3). Postprandial changes in the plasma glucose concentration serve as a cue for secretion of insulin by the β -cells in islets of Langerhans in pancreas. Insulin stimulates the uptake of glucose in insulin responsive tissues like muscle and the adipose tissue. It promotes storage of metabolic substrates as lipids or glycogen and inhibits hepatic gluconeogenesis. It also inhibits the release of FFAs by inhibiting lipolysis and glycogenolysis thereby tightly regulating the circulating levels of glucose and lipids in the blood. Adipose tissue accounts for about 10-15% of post prandial glucose uptake while the rest is taken up by the muscle. Adipokines released from adipocytes can have both positive and negative effects on glucose homeostasis. Adipokines like leptin and adiponectin improve glucose homeostasis, whereas tumor necrosis factor α (TNF α) and retinol binding protein 4 (RBP4) are known to impair insulin action [reviewed in (4)]. Resistance to insulin leads to elevated levels of glucose in the circulation also known as hyperglycemia, and perturbed levels of fatty acids, triglycerides and lipoproteins, also known as dyslipidemia; both of which are characteristics of type 2 diabetes (T2D).

INSULIN RESISTANCE AND TYPE 2 DIABETES

Type 2 diabetes is a metabolic disorder characterized by hyperglycemia resulting from insulin resistance and/or insufficient insulin secretion, and is caused by a complex interaction of lifestyle, diet and genetics. The International Diabetes Federation (IDF) projects that the prevalence of diabetes will expand from the current estimate of 415 million to 642 million people by 2040, of which greater than 90% have type 2 diabetes (5). The ever increasing urbanization, unhealthy diet and sedentary lifestyle have been fueling this ‘tsunami of diabetes’. Transformed into one of the largest global epidemics, diabetes is one of the top ten leading causes of death in the world (6). Together with its associated complications such as cardiovascular diseases, neuropathy, nephropathy and retinopathy, it poses a huge financial burden on the healthcare systems.

According to IDF, T2D is diagnosed if one or more of the following criteria are met:

- a) Fasting plasma glucose ≥ 7.0 mmol/L (126 mg/ dl)
- b) Two-hour plasma glucose ≥ 11.1 mmol/L (200 mg/dl) following an oral glucose tolerance test (OGTT)

Metabolic syndrome

A cluster of risk factors for T2D and cardiovascular disease such as central obesity, dyslipidemia, hypertension and hyperglycemia have been coined as the metabolic syndrome.

OBESITY AND METABOLIC HOMEOSTASIS

Obesity and overweight significantly increases the risk of T2D and are one of the major contributors to the increased prevalence of T2D. According to WHO,

overweight is defined as a body mass index (BMI) of greater than or equal to 25 kg/m² and obesity is defined as BMI greater than or equal to 30 kg/m². Obesity is associated with remodeling and expansion of the adipose tissue. The expansion can occur either via hyperplasia i.e. increase in number of adipocytes, or by hypertrophy i.e. enlargement of the existing adipocytes. Impaired adipose tissue remodeling in the obese state can occur due to reduced angiogenesis, increased extracellular matrix production, increased macrophage infiltration and inflammation, all of which contribute to insulin resistance. Healthy expansion of the adipose tissue is thus important to avoid the lipotoxic effects and to preserve the systemic insulin sensitivity (7). Not all obese individuals are insulin resistant which indicates that the metabolically healthy individuals are capable of healthy expansion of adipose tissue - thus arises the concept of “metabolically healthy obese” individuals (8-10).

Lipotoxicity

Lipotoxicity refers to the pathological condition that may occur when FFA flux in excess of the oxidative needs of a tissue, promotes metabolic flux into harmful nonoxidative pathways.

The number of adipocytes in an individual appears to be set during childhood and adolescence and remains constant in adulthood, however adults exhibit a turnover of 10% fat cells annually (11). There is a strong correlation between the size and insulin sensitivity of adipocytes (12-15). For example, large adipocytes exhibit reduced glucose uptake and enhanced lipolysis compared to the small adipocytes from the same individual (12, 13) and also have an altered gene expression profile (16). Insulin sensitive obese individuals have higher number of small adipocytes compared with insulin resistant obese individuals (14) and individuals with hypertrophic adipocytes, regardless of obesity have an increased risk of developing T2D in future (17, 18). Also, improved insulin sensitivity observed after substantial weight reduction is associated with decrease in fat cell volume (19). Thus, healthy adipose tissue remodeling and developmental pathways that regulate recruitment and differentiation of fat cell appear to be crucial in determining the metabolic state in obesity.

INSULIN RESISTANCE AND ADIPOSE TISSUE DYSFUNCTION

Insulin resistance is a state in which the insulin responsive tissues exhibit diminished response to normal concentrations of insulin. In order to compensate for insulin resistance and maintain normal glucose concentrations, the β -cells produce more insulin but eventually fail to do so and T2D can be diagnosed. The insulin resistant state is also sometimes called pre-diabetes. Insulin resistance is a hallmark of T2D, but one must keep in mind that not all insulin resistant individuals become diabetic.

Adipose tissue research has gained more limelight over the years and it is being more widely accepted that insulin resistance originates due to adipose tissue dysfunction. Adipose tissue dysfunction is a result of complex interaction of genetics, lifestyle and environmental factors which can lead to adipocyte hypertrophy, hypoxia, impaired mitochondrial function and inflammatory processes within the adipose tissue (Figure 2). Absence of appropriate adipose tissue expansion in times of energy excess leads to lipid spill-over leading to abnormal lipid deposition in other organs (ectopic fat accumulation) thus spreading insulin resistance to organs such as liver, skeletal muscle, pancreas and heart [reviewed in (7, 20, 21)]. Described below are some possible mechanisms how dysfunctional adipose tissue can lead to insulin resistance.

Inflammation

Low grade chronic inflammation in obesity is associated with insulin resistance (22-24). However, the molecular events leading to initiation of inflammatory response in the adipose tissue is still unknown. Unhealthy expansion of the adipose tissue can lead to a plethora of effects including hypoxia due to impaired angiogenesis, adipocyte cell death, fibrosis and dysregulated fatty acid flux (Figure 2). A short exposure to high fat diet for just a few days can cause adipocyte hypertrophy and initiation of hypoxic responses in the adipocytes (25). The hypoxic micro-environment due to adipocyte hypertrophy in obese state has been shown to upregulate many pro-inflammatory adipokines like migration inhibitory factor (MIF), matrix metalloproteinases MMP1 and MMP2, IL-6, leptin, and vascular endothelial growth factor (VEGF). But on the other hand, it can downregulate the anti-inflammatory adipokine, adiponectin (26-28). The size of the adipocytes by itself has been shown to affect the adipokine gene expression profile. The hypertrophic adipocytes can secrete increased pro-inflammatory

adipokines (16, 29) and many of those like TNF α , monocyte chemoattractant protein 1 (MCP-1) and interleukins IL-6, IL-1 and IL-8 have been shown to promote insulin resistance (30-32).

Figure 2: Hypertrophic adipocytes are associated with reduced angiogenesis, increased macrophage infiltration, inflammation and altered adipokine expression. The consequent lipid spill over from the adipocytes leads to ectopic fat deposition and hence systemic insulin resistance. Dysfunctional adipose tissue thus creates a milieu that promotes insulin resistance, in the adipose tissue and systemically.

In advanced obesity, adipocyte hypertrophy increases adipocyte death which stimulates the infiltration of pro-inflammatory macrophages surrounding the necrotic adipocytes in the adipose tissue (33-35), which further contributes to the inflammatory process by releasing pro-inflammatory cytokines. It has also been shown that increased macrophage infiltration in obesity transforms the polarized state of the macrophages from an anti-inflammatory M2 polarized state to pro-inflammatory M1 polarized state (36, 37). Studies also show that M1 population

leads to increase in pro-inflammatory cytokines that contribute to insulin resistance (38, 39). On the other hand, M2 macrophages promote adipose tissue remodeling by clearance of dead adipocytes, promoting uptake and oxidation of FFA, and recruitment and differentiation of adipocyte progenitors (40). Increased levels of circulating FFAs associated with obesity (41) can also promote adipose tissue macrophage recruitment (42), and can induce inflammatory signaling pathways like c-Jun N-terminal kinase (JNK), nuclear factor-kappaB (NFκB) and toll-like receptor (TLR) signaling. These inflammatory signaling pathways in turn have been shown to contribute to the development of insulin resistance (43-45).

TNFα is another pro-inflammatory adipokine demonstrated to be increased in obese rodent models of insulin resistance, as well as in obese humans (30, 46). It is believed to be released from the activated adipose tissue macrophages (47). However, opposed to the prevalent notion that inflammation induces insulin resistance, Asterholm *et al.* in a recent study showed that acute inflammatory response in the adipose tissue is essential for adipose tissue protection, remodeling, and expansion. They showed that abrogation of acute inflammatory response in the adipose tissue impaired adipogenesis, followed by ectopic fat accumulation and glucose intolerance under high fat diet exposure (48).

Whether inflammation is a cause or a consequence for insulin resistance is still debatable (48-50). One might say in light of these studies that inflammation could be part of the physiology of adipose tissue expansion and acts as a defensive mechanism until it turns into a chronic phenomenon and causes insulin resistance (51). Also, adipocyte hypertrophy by itself can cause insulin resistance in a cell autonomous manner independent of inflammation as suggested by Kim *et al.* (52).

Impaired adipogenesis

Expansion of the adipose tissue can occur by recruiting newly differentiated adipocytes by hyperplasia or by enlargement of existing adipocytes by hypertrophy. Differentiation of adipocytes is a complex process that involves commitment into pre-adipocytes, mitotic clonal expansion and finally terminal differentiation into mature adipocytes. The differentiation process requires coordinated action of several transcription factors of which peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer binding protein alpha (C/EBPα) are the most important (53, 54). Pre-adipocytes in

hypertrophic obesity exhibit reduced differentiation potential which appears to be due to impaired ability to recruit and differentiate into new adipocytes and not due to reduced early adipocyte precursor pool (55). Overcoming impaired adipogenesis by inhibition of WNT signaling pathway or by activation of bone morphogenetic protein 4 (BMP4) further supports this as crosstalk between these two regulates the adipogenic commitment and differentiation (56, 57). Impaired adipogenesis can therefore lead to development of insulin resistance by causing adipocyte hypertrophy, inflammation and ectopic fat deposition.

Genetics and epigenetics

Insulin resistance and impaired insulin secretion are the two major characteristics of T2D and insulin resistance is highly correlated with obesity. Therefore, accumulation of risk alleles for obesity, insulin resistance or impaired insulin secretion predisposes individuals to T2D. There is ample evidence of hereditary predisposition for T2D such as increased risk for first degree relatives of individuals with T2D (58) and increased risk for certain populations like Pima Indians and South Asians (59, 60). Among the genetic variants that increase T2D risk, most of the associated loci are mapped to genes related to β -cell dysfunction rather than insulin resistance (61, 62). As a result, knowledge about the genetic basis of insulin resistance is rather limited.

Some of the genes linked to T2D that have been consistently replicated in linkage studies as well as genome wide association studies include TCF7L2, PPARG and KCNJ11 (63, 64). TCF7L2 is a member of the LEF/TCF family of transcription factors and is a known regulator of WNT signaling pathway. It was initially known to play a role in pancreatic function but there is growing evidence of its role in liver and possibly in adipose tissue (65-67). In the adipose tissue, inhibition of WNT signaling is critical for differentiation of adipocytes. PPARG on the other hand, is known to positively regulate adipogenesis. Thus, cross-talk between PPARG and WNT signaling, which has also been shown in adipocytes is likely to play an important role in regulating the differentiation of adipocytes [reviewed in (68)]. It is interesting that the two most established genetic variants PPARG and TCF7L2 are involved in regulation of adipogenesis, which further strengthens the notion of impaired adipogenesis as an important factor in development of insulin resistance and T2D. Only few genes involved in insulin action have been shown to be associated with T2D, some of which are IRS1, PPARG and ADIPOQ (64, 69). IRS1 was among the five loci found to be

associated with fasting insulin in the blood as well as a dyslipidemic profile, strengthening its role in insulin resistance (70). The genetic variants of IRS1 can result in insulin resistance not only due to its role in insulin signaling but also in adipocyte differentiation (71). A large number of genes associated with T2D still have unknown functions indicating many unexplored aspects of T2D. Understanding the functional significance of the identified genes can help us to further understand the pathophysiology of T2D.

It is interesting that genetic variants explain only a small proportion of the observed heritability of T2D. This brings into focus the role of epigenetics and variations in the non-coding parts of the genome in development of T2D, the latter of which has just started to being explored (72). Exposure to famine during first half of the pregnancy, for example, was found to be associated with obesity in adult offspring in a historical cohort study of 19-year old Dutch men (73). Also, changes in maternal body composition before or during pregnancy seem to be related to adiposity of the offspring (74). Furthermore, maternal diet during pregnancy has been suggested to influence neonatal adiposity (75) and has also been shown to induce epigenetic modifications that can be reverted by diet intervention during pregnancy (76).

Epigenetic changes have also been reported in human adipocytes. While a global reduction of dimethylation of histone H3 at lysine 4 (H3K4me2) has been observed in adipocytes from overweight and type 2 diabetic subjects as compared to normal weight subjects, an increase in trimethylation of H3 at lysine 4 (H3K4me3) was observed in type 2 diabetic subjects as compared to normal and overweight subjects (77). In line with this, it is interesting that mice with mutations in H3K4 methyltransferase MLL3, displayed decreased adiposity and increased insulin sensitivity as compared to control mice (78). Also, increase in H3K4me3 at PPAR γ promoters appears to be associated with increased PPAR γ expression during adipogenesis in 3T3-L1 adipocytes (79).

Hyperglycemia is another factor that can induce long-term epigenetic changes and it has been shown that cells derived from hyperglycemic environment retain 'hyperglycemic memory' even when they are returned to normal glucose environment (80, 81). It has also been suggested that histone methyltransferase (SET7) and a lysine-specific demethylase (LSD1) might be involved in mediating epigenetic changes during transient hyperglycemia (82).

In a very recent study, Multhaup *et al.* (83) identified differentially methylated regions (DMRs) in subcutaneous adipose tissue between lean subjects and age- and sex-matched insulin resistant obese individuals pre- and post-Roux-en-Y gastric bypass (RYGB). A number of DMRs were shown to overlap with known T2D risk loci. One of the interesting findings was that after gastric bypass surgery the methylation pattern for a number of loci identified in obese individuals changed towards that of samples from lean individuals. This indicates that at least some of the epigenetic modifications in adult obesity are reversible. Functional analysis of some of the genes with reversed methylation after RYGB, and with no prior association with any metabolic phenotype, indicated their possible role in insulin resistance. Epigenetic mechanism in T2D is a relatively new area of research. There is much to be explored in this field and has prospects of answering many unresolved questions.

INSULIN SIGNALING

Insulin stimulation of the adipocytes initiates a cascade of events that precipitate into control of a number of cellular processes such as glucose uptake, protein synthesis, autophagy and inhibition of lipolysis. Insulin signaling pathways involve numerous cross-talks, feedbacks (both positive and negative), protein interactions and modifications, and proper subcellular localization, which merely gives a sneak peek into the remarkable complexity of these pathways [reviewed in (3, 84)].

Through a series of publications, we have elucidated the molecular details of different parts of the insulin signaling pathways in primary human adipocytes. Insulin receptor signaling involves two major pathways, phosphatidylinositol 3-kinase (PI3K) pathway and mitogen activated protein kinase (MAPK) pathway (Figure 3). The parts marked as gray in figure 3 indicate the focus of this thesis. Key metabolic effects of insulin are mediated via PI3K-PKB signaling and the key mitogenic effects are mediated via MAPK signaling. Our research group has previously described the initial stages of insulin signaling i.e. insulin binding, receptor internalization and recycling (85) as well as insulin control leading to glucose uptake, protein synthesis and autophagy, normally and in T2D (86, 87).

We employ a unique approach of combining consistently obtained experimental data with mathematical modeling to build a mathematical model of the insulin signaling network, normally and in T2D. This has allowed us to elucidate mechanisms that can convert insulin signaling in normal individuals into that of those with T2D. Through paper I-III, we further expand our understanding into insulin regulation of MAPK signaling for transcriptional control and transcriptional control by FOXO1 in adipocytes, and how these pathways are dysregulated in T2D. To understand the molecular details of how these pathways work, it is important to build on the current knowledge about the pathogenesis of insulin resistance and type 2 diabetes and identify opportunities for drug intervention and treatment. This section will give a background on key elements of the insulin signaling pathways.

Figure 3: Insulin signaling network in the human adipocytes. P denotes the phosphorylation sites and the green arrow indicates the positive feedback signal from mTORC1 to IRS1. The focus of this thesis is marked in gray.

RECEPTOR ACTIVATION

The insulin receptor (IR) is a disulfide-linked heterotetramer, consisting of two extracellular α -subunits and two transmembrane β -subunits, where α -subunit exerts inhibitory influence on the tyrosine kinase activity of the β -subunit (Figure 4). Binding of insulin to the specific regions of the α -subunit relieves this repression and leads to rapid conformational changes in the receptor. This induces auto-phosphorylation of the cytoplasmic tyrosine residues in the β -subunit and hence activation of its tyrosine kinase activity. Activated IR can then phosphorylate tyrosine residues of substrate proteins such as insulin receptor

substrate-1 (IRS1) and Shc, which propagate the signaling further downstream (3).

IR is localized to the caveolae (88-90), which are invaginations in the plasma membrane that act as signaling platforms in adipocytes. Following activation, insulin receptor is rapidly internalized through caveolae mediated endocytosis where insulin dissociates from IR and the receptor is dephosphorylated and recycled back to the cell surface (91, 92).

ACTIVATION OF IRS1 AND PI3K

Insulin receptor substrate (IRS) proteins are direct downstream effectors of IR and act as a scaffold to bring together signaling complexes. Out of the four isoforms of IRS, IRS1 is the major isoform in the muscle and adipose tissue. IRS1 and IRS2 have complementary roles in liver, meanwhile IRS4 seems dispensable for glucose homeostasis (93, 94). IRS3 on the other hand, is only present in rodents and not in humans (95). These isoforms not only have tissue-specific expression but also tissue-specific functions (96).

Figure 4: A schematic illustration of activation of IR, IRS1 and PI3K. Tyrosine phosphorylated residues of IR bind the PTB domain of IRS1. Tyrosine phosphorylated IRS1 recruits PI3K via SH-2 domain. PI3K converts PIP2 to PIP3 that can then recruit PKB to the plasma membrane via PH domain.

IRS1 is recruited by the activated IR through pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, and is phosphorylated by the activated IR at multiple tyrosine residues. Phosphorylated IRS1 subsequently serves as a docking site for downstream signaling proteins containing Src-homology 2 (SH2) domains like PI3K. Once PI3K is recruited to the membrane, it increases the concentration of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) that can then bind PH domain containing proteins like protein kinase B (PKB), as shown in figure 4 (84).

REGULATION OF INSULIN SIGNALING THROUGH FEEDBACKS VIA IRS1

IRS1 also has many insulin responsive serine and threonine phosphorylation sites that have been shown to participate in both positive and negative regulation of insulin signaling [reviewed in (97)]. In general, serine/threonine phosphorylations are considered to negatively affect insulin signaling by either triggering proteasomal degradation of IRS1 or by disrupting the interaction between IR and IRS1. A number of kinases have been implicated in mediating these phosphorylations such as atypical PKC, PKB, mTOR, S6K1, ERK1/2, JNK and IKK β [reviewed in (98)]. In contrast to this, we have shown that insulin-induced phosphorylation of IRS1 at Ser³⁰⁷ (human sequence corresponding to Ser³⁰² in mouse) is involved in a positive feedback control in adipocytes (99-102), though the contrary has been reported in animal models and other cell lines (103, 104). What makes this site really interesting is that its phosphorylation is also reduced in adipocytes from diabetic individuals (86). In fact, using a combined experimental and modeling approach, we have shown that attenuation of this feedback explains most of the insulin resistance in adipocytes from diabetic individuals [(86), Paper I and Paper II] creating an interest to identify the responsible protein kinase. It is clear that the responsible kinase lies downstream of mTORC1 (99, 105, 106) and both mTORC1 and its downstream substrate p70S6 kinase-1 (S6K1) have been suggested as the kinase (104, 105, 107). However, we have shown that both S6K1 and mTOR are unlikely to be the physiological kinase for IRS1 Ser³⁰⁷, although a potential candidate protein kinase immunoprecipitated with mTOR in insulin-stimulated lysates of human adipocytes (108). However, the identity of the protein kinase in the mTOR-immunoprecipitate remains to be identified.

Another positive feedback via Ser³²³ (human sequence corresponding to Ser³¹⁸ in mouse) has been reported in muscle cell line. The investigators suggested that it is the pattern of phosphorylation and interaction between different phosphorylation sites rather than phosphorylation of one site that determines the positive or negative regulation (102). This might also explain why some of the sites have been implicated in both positive and negative regulation of insulin signaling. Phosphorylation of Ser³²³ might regulate the insulin signaling positively in human adipocytes as it was found to be reduced in adipocytes from T2D individuals (unpublished data).

Ser³¹² (human sequence corresponding to Ser³⁰⁷ in mouse) on the other hand, was for a long time believed to be a negative regulatory site that was shown to block interaction between IR and IRS1 (97). However, knock-in mice in which Ser³¹² was replaced with alanine, developed severe insulin resistance under high fat diet as compared to the control mice (109). Furthermore, mice with genetic knock-in of a serine-to-alanine mutation for Ser³⁰⁷ exhibited normal insulin signaling and glucose homeostasis (110). These studies re-emphasize the importance of evaluating physiological interpretations when translating findings from *in vitro* to *in vivo* conditions in animal models and then further into humans.

ACTIVATION OF PKB

Protein kinase B (PKB) also known as Akt exists in the three isoforms PKB $\alpha/\beta/\gamma$ or Akt 1/2/3. PKB α is expressed ubiquitously. PKB β is abundant in the insulin responsive tissues like adipose tissue, liver and skeletal muscle, whereas PKB γ is highly expressed in brain (111-113). PIP3 generated by PI3K, recruits PKB and phosphoinositide-dependent kinase-1 (PDK1) via their PH domains to the plasma membrane. Once recruited at the plasma membrane, PKB is phosphorylated at Thr³⁰⁸ by PDK1 (114-116) and at Ser⁴⁷³ by mTORC2 (117) (Figure 5).

Phosphorylation of both sites is required for optimal activation of PKB. There are conflicting findings about the sequence of phosphorylation of the two sites. Some of the earlier studies show that the prior phosphorylation of PKB at Ser⁴⁷³ has been shown to facilitate phosphorylation of PKB at Thr³⁰⁸ (118, 119), although some studies also report the two sites to be independent of each other (116, 120). Additionally, a recent study by Yang *et al.* suggested that phosphorylation of

PKB at Thr³⁰⁸ is required for SIN1 phosphorylation, a key component of mTORC2 complex. They showed that PDK1 phosphorylation of PKB at Ser³⁰⁸ is required for phosphorylation of SIN1 at Thr⁸⁶ in mouse embryo fibroblasts (MEFs), which in turn increases mTORC2 activity and hence phosphorylation of PKB at Ser⁴⁷³ (121).

Figure 5: Full activation of PKB requires phosphorylation of threonine 308 by PDK1 and the phosphorylation of serine 473 by mTORC2.

The two phosphorylations together may enhance the kinase activity by about 1000-fold (116) and singly phosphorylated PKB at Thr³⁰⁸ has been shown to be active but is said to be a weaker enzyme (122). Another recent study showed that phosphorylation of two additional sites, at Ser⁴⁷⁷ and Thr⁴⁷⁹, can promote the interaction of PKB with mTORC2 complex and facilitate phosphorylation at Ser⁴⁷³ or even functionally compensate for phosphorylation at Ser⁴⁷³ by locking PKB in its active conformation (123). PKB activation is a complex process and layers of regulation still remain to be unraveled.

PKB acts as a major hub in the insulin signaling pathway and regulates many cellular processes (Figure 6). It controls glucose uptake via phosphorylation of AS160 that regulates the glucose transporter GLUT4. It activates mTORC1,

another major node in the insulin signaling network that regulates many other cellular processes (discussed in next section). It also activates glycogen synthesis through inhibition of glycogen synthase kinase-3 (GSK-3) and inhibits transcriptional activities mediated by FOXO1.

Figure 6: PKB regulates number of cellular processes in the cell like glucose uptake, transcription, protein synthesis and glycogen synthesis. It also regulates mTORC1, another major signaling hub in the cell.

So what impact does phosphorylation of PKB by two different kinases that differ markedly in their regulation, have on its substrates? Knockout studies have shown that mTORC2 inactivation differentially affects substrates of PKB. For example, insulin-induced phosphorylation of FOXO1/3a but not GSK-3, tuberous sclerosis

complex 2 (TSC2), p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) was impaired in rictor-, mLST8- or SIN1-deficient MEFs (122, 124). PKB phosphorylated at Thr³⁰⁸ has been suggested to primarily regulate protein synthesis via mTORC1 activation, whereas PKB phosphorylated at Ser⁴⁷³ has been suggested to regulate glucose uptake via phosphorylation of AS160 and regulate FOXO1 activity (125, 126). Although, another study in rat adipocytes indicated that PKB phosphorylated at Thr³⁰⁸ and not Ser⁴⁷³, is associated with glucose uptake (127). The two phosphorylation sites of PKB at Ser⁴⁷³ and Thr³⁰⁸, thus possibly dictate the functional dissection of PKB in the cells, which makes it possible for PKB to control such diverse functions.

MAMMALIAN TARGET OF RAPAMYCIN (mTOR)

mTOR is one of the master regulators of metabolism and growth that senses the local and systemic fluctuations of nutrients and energy in the cell. It integrates the information from various intracellular and extracellular cues and relays it to downstream mediators to regulate cell growth and differentiation, lipid and protein synthesis, autophagy, mitochondrial functions, and other metabolic processes. mTOR exists in two structurally and functionally distinct multiprotein complexes mTORC1 and mTORC2. They differ not only in their sensitivity to the immunosuppressant rapamycin but also in their composition, regulation and function. mTOR is the catalytic subunit in both the complexes. Its interaction partners and the subcellular localization of the two complexes regulate the activity and substrate specificity of the two complexes [reviewed in (128, 129)]. Dysregulation of mTOR is implicated in many diseases including obesity, T2D and several types of cancer and has therefore been a subject of intensive research.

mTORC1

mTORC1 is composed of mTOR, regulatory associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL); proline-rich AKT substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor). In presence of growth factors or amino acids, mTORC1 translocates to the lysosomes where it is activated by the GTPase Ras

homolog enriched in brain (Rheb). However, mTORC1 has also been observed in other subcellular locations such as mitochondria and plasma membrane [reviewed in (130)]. The GTPase activity of Rheb is inhibited by TSC1/2 complex which functions as GTPase-activating protein (GAP) for Rheb. Growth factor stimuli results in phosphorylation of TSC2 by PKB, which relieves the repression of Rheb. Activated Rheb then activates mTORC1 (Figure 7). PRAS40 is a negative regulator of mTORC1. PKB can also activate mTORC1 in TSC1/2 independent manner by phosphorylating PRAS40 and promoting its dissociation from mTORC1 (131, 132).

Figure 7: PKB activates Rheb by relieving the repression by TSC1/2 complex. Active Rheb phosphorylates and activates mTORC1. S6K1 and 4E-BP1 are two major targets of mTORC1 that regulate protein synthesis. mTORC1 also regulates autophagy and mitochondrial function.

mTORC1 is the rapamycin sensitive complex, however not all downstream substrates of mTORC1 are sensitive towards rapamycin. It has been suggested

that mTORC1 phosphorylation sites that are poor *in vitro* substrates such as S6K1 Thr³⁸⁹ are more sensitive to rapamycin as compared to good *in vitro* substrates such as 4E-BP1. Also, rapamycin may be more effective towards substrates that require the FKBP12-rapamycin-binding domain for interaction with mTORC1 [reviewed in (133)].

Two main downstream substrates of mTORC1 are S6K1 and 4E-BP1, which positively regulate protein synthesis. mTORC1 also phosphorylates unc-51-like kinase 1 (ULK1) and autophagy-related gene 13 (ATG13) to inhibit autophagy (134). Constitutive activation of mTORC1 in TSC2-deficient MEFs resulted in impaired autophagy, whereas knockdown of raptor or treatment with rapamycin, activated autophagy in TSC2-deficient MEFs (135). Another important function of mTORC1 is to regulate mitochondrial biogenesis and metabolism. It seems to have both positive and negative effects on mitochondrial function and the effects seem to vary depending on tissue type. For instance, adipose-specific raptor knock-out exhibited enhanced mitochondrial respiration (136), while muscle-specific raptor knock-out resulted in decreased oxidative capacity and reduced expression of genes involved in mitochondrial biogenesis (137).

mTORC2

mTORC2 is comprised of mTOR; rapamycin-insensitive companion of mTOR (Rictor); mammalian stress-activated protein kinase interacting protein 1 (mSIN1); protein observed with Rictor-1 (Protor-1), mLST8, and deptor. A specific subcellular localization of mTORC2 has not been demonstrated. It has been found in the plasma membrane in different cell types together with its substrate PKB, which is known to be activated at the plasma membrane. However, recent studies indicate that mTORC2 is localized in the mitochondria-associated ER membranes (MAMs) which are subdomains of ER physically associated with mitochondria. Known substrates of mTORC2 are phosphorylation of PKB at Ser⁴⁷³, serum and glucocorticoid-induced kinase (SGK) and protein kinase C (PKC) [reviewed in (130, 138)]. However, as compared to mTORC1, much less is known about the regulation and function of mTORC2 in the cell. mTORC2 plays an important role in glucose and lipid metabolism, which is evident from mice models with adipose- or muscle-specific knockout of rictor. Genetic deletion of rictor in adipose tissue caused impaired insulin-stimulated GLUT4 translocation and hence decreased glucose transport. They also exhibit

increased levels of circulating FFA due to increased lipolysis and developed hepatic steatosis and insulin resistance in liver as well as skeletal muscle (126). Muscle-specific rictor knockout mice on the other hand, were glucose intolerant and displayed reduced insulin-stimulated glucose uptake in muscle (139). Also, liver-specific rictor knockout mice displayed increased gluconeogenesis and impaired lipogenesis (140).

mTORC2 complex is insensitive to rapamycin, although prolonged exposure to rapamycin has been shown to disrupt mTORC2 assembly and thus inhibit mTORC2 in some cell types, such as HeLa or PC3 cells. Treatment of these cells with rapamycin for 24 hours dramatically reduced rictor-mTOR association and consequently phosphorylation of PKB at Ser⁴⁷³ (141). We did however, not observe any effect of rapamycin on mTORC2 activity in human adipocytes even after 48 hours with rapamycin, as observed from insulin-induced phosphorylation of PKB at Ser⁴⁷³ (Paper II).

RESULTS AND DISCUSSION

ERK SIGNALING IN HUMAN ADIPOCYTES

Apart from controlling metabolic responses via the PI3K-PKB pathway, insulin also regulates mitogenic responses via the MAPK pathway. The MAPK family consists of extracellular signal regulated kinase 1/2 (ERK), p38 protein kinase (p38), c-Jun N-terminal kinase family (JNK) and several more. While p38 and JNK typically respond to cellular stress, ERK is activated in response to growth factors and also hormones like insulin. ERK is encoded by two genes ERK1 and ERK2 which are 84% identical in sequence and share many functions. ERK orchestrates a variety of cellular functions such as cell growth and differentiation, transcription and cell cycle progression [reviewed in (142)]. Insulin signaling through ERK is relatively under-investigated and very little is known about this branch of insulin signaling, especially in T2D. In Paper I, we have therefore examined the mitogenic signaling by insulin and build upon our previously developed model of metabolic branch of insulin signaling. In this section, I will discuss how our findings have added to our understanding of insulin regulation of ERK in the human adipocytes, both normally and in T2D.

INSULIN REGULATED PHOSPHORYLATION OF ERK

Activation of the ERK pathway mainly occurs at the plasma membrane where, in response to insulin, activated IR recruits IRS1 and/or Shc (143) or any other adaptor protein containing SH2 or PTB domain. The adaptor protein then binds the growth factor receptor-bound protein 2 (Grb2) and recruits the guanine exchange factor, SOS to the membrane. This promotes the interaction of SOS with the membrane localized small GTPase Ras. Activated Ras activates the protein kinase Raf that then phosphorylates and activates the protein kinase MEK, which in turn phosphorylates and activates ERK (142). Phosphorylated ERK translocates into the nucleus where it can phosphorylate its nuclear targets like Ets-like gene-1 (Elk1) (144, 145) (Figure 8).

ERK activation has been shown to be mediated by IRS1-PI3K pathway in 3T3-L1 adipocytes as well as in rat adipocytes where insulin-induced phosphorylation of ERK was reduced in the presence of the PI3K inhibitor wortmannin (146, 147). In line with this, we have shown previously that ERK phosphorylation in response to insulin in human adipocytes is mediated by IRS1 since adipocytes transfected with IRS1 mutants displayed reduced Elk1 phosphorylation in response to insulin (148). On the other hand, other studies report involvement of both IRS1 and Shc but with predominant contribution of Shc in insulin-induced activation of ERK (149, 150).

Figure 8: Insulin mediated phosphorylation of ERK can occur via IRS1 and/or Shc. The subsequent signaling cascade involves phosphorylation of Ras, c-Raf, MEK and ERK. Phosphorylated ERK translocates into the nucleus to activate the transcription factor Elk1.

Depending on cell type or agonist, difference in the phosphorylation kinetics of ERK has been observed, which can be either transient or sustained. It is also really fascinating how these different phosphorylation kinetics of ERK induce

different cellular responses. For example, nerve growth factor stimulation of PC12 cells, a neuronal differentiation cell model, produced a sustained phosphorylation that lead to differentiation, while epidermal growth factor (EGF) stimulation of PC12 cells induced a transient activation of ERK that lead to cell proliferation (151).

In paper I, we examined the dynamics of ERK phosphorylation in response to insulin. We show that insulin-induced phosphorylation of ERK is transient peaking around 10 min and returning back to basal level by 60 min (Figure 9). Differences in cell type, tissue or species have been shown to affect the dynamic behavior of ERK phosphorylation. For example, human adipocytes display a much smaller response to insulin (around 2-fold) with maximum after 10 minutes (Paper I), as compared to murine-derived 3T3-L1 adipocytes with 18-fold response and maximum at around 2 minutes (152). Moreover, different kinetics of ERK phosphorylation have been observed in mice liver and muscle tissues that exhibit sustained phosphorylation, whereas primary mice adipose tissue showed transient phosphorylation of ERK (153). We were further intrigued by how the upstream signal from tyrosine phosphorylation of IRS1, which exhibited a rapid overshoot and an elevated steady state level, converted into that of a transient response in ERK phosphorylation in human adipocytes [(86), Paper I] (Figure 9). We addressed this by using mathematical modeling to investigate the possible mechanisms that may explain the observed dynamics for ERK phosphorylation in human adipocytes (Paper I).

Figure 9: Comparison of dynamics between insulin-induced ERK and IRS1 phosphorylation. ERK phosphorylation is slower and comes back to the basal level, whereas IRS1 phosphorylation is rapid and displays an elevated steady state level.

MATHEMATICAL MODELLING AND HYPOTHESIS TESTING

Mathematical modeling, also referred to as systems biology, is a quantitative method to study the structure and kinetics of a biological system and it enables us to examine signaling networks as a whole rather than focusing on one part at a time. It provides us means for data analysis and hypothesis testing, as well as to predict the most informative experiments (154).

In order to construct a mathematical model, possible mechanisms are first enlisted based on the insights from the prior knowledge of the system and available experimental data. These mechanisms/hypotheses are then translated into mathematical equations. When data contains kinetic information, such as a time-response to an input, the choice of mathematical equations is usually ordinary differential equations (ODEs). ODEs capture the kinetics of a biological system. ODEs include ‘states’ that correspond to the concentration or amount of the proteins in the model; ‘parameters’ that correspond to rate constants that are usually estimated from the model and experimental data, keeping in mind the realistic limits of the parameter; and ‘output variables’ that correspond to measured experimental data (155).

Minimal Model
Minimal models describe the key components of the systems functionality and are physiologically based models that can describe the kinetics of a dynamic system with smallest number of identifiable parameters (Cobelli et al. 2009).

When evaluating a developed model’s ability to describe a biological system, a common method is to simulate a non-tested experimental setup with the model. This simulation is a prediction that can be tested experimentally to confirm the accuracy of the developed model. To account for the uncertainty in data in such a prediction, we gather all parameters that are in agreement with experimental data according to a χ^2 test. Prediction that are simulated using all these parameters, and at the same time show a unique property that can be tested experimentally, are known as core predictions. Core predictions can be used for model/hypothesis rejections (156). Hypothesis testing with minimal models was used to test different hypotheses that could explain the transient dynamics of ERK

phosphorylation in response to insulin. The activity and specificity of ERK signaling is regulated by different features which form the basis of our hypotheses. Described below are some of these features.

Degradation

Sorbitol-induced osmotic stress has been shown to ubiquitinate ERK and target it to proteasomal degradation in NIH3T3 and HEK293T cells via E3 ubiquitin ligase activity of MEKK1 (MEK kinase) towards ERK2. Thus, stress but not serum or EGF stimulation has been shown to induce ERK degradation (157). A minimal model for degradation of ERK can indeed explain the transient dynamics of ERK phosphorylation. A search for parameters showed that for the model to statistically fit with the data, 40% of ERK should be degraded after 90 min. We tested this experimentally and found that only 10% of ERK was degraded after 90 min and could thus reject our hypothesis of ERK degradation as a possible mechanism (Paper I).

Subcellular localization

The subcellular localization of various components of this cascade is one of the important factors that regulate the activity and specificity of this pathway. Raf kinase translocates to the plasma membrane upon stimulation for activation by Ras, following which it can phosphorylate and activate MEK1/2. MEK1/2 is a dual specificity kinase that phosphorylates ERK at tyrosine and threonine residues and has been shown to shuttle between the cytoplasm and the nucleus. Inactive ERK can be bound to cytoplasmic anchor proteins where dual phosphorylation of ERK by MEK1/2 can induce a conformational change that releases phosphorylated ERK from its anchoring proteins. Phosphorylated ERK is known to translocate to cytoplasmic organelles like endosomes and golgi apparatus, however, nucleus is known to be the main translocation target for ERK [reviewed in (158)].

Nuclear sequestration of phosphorylated ERK and its dephosphorylation by specific nuclear phosphatases like MAPK phosphatases (MKPs) has been demonstrated (144). Consistent with this, in paper I we observed a strong nuclear localization of ERK in the human adipocytes with confocal microscopy. A minimal model for nuclear translocation of ERK, where ERK must be

dephosphorylated before going back into the cytoplasm, could explain the observed dynamics for ERK phosphorylation in human adipocytes (Figure 10).

Scaffolding

Scaffolding proteins also play a very crucial role in the ERK signaling cascade in regulating the specificity of the signaling. They bring the signaling components together and facilitate propagation of the signal, targeting of the signal to specific cellular compartments or mediating cross-talks with other pathways. Some of the well-known scaffolding proteins involved in ERK signaling are kinase suppressor of Ras 1 (KSR1), IQ motif containing GTPase activating protein 1 (IQGAP1) and β -arrestin [reviewed in (142)]. KSR1 has been shown to coordinate the formation of RAF-MEK-ERK complex and relays the signal transmission from c-Raf to MEK to ERK (159, 160). Binding of IQGAP1 to B-Raf, MEK1/2 and ERK and their respective ratio has been shown to be critical for optimal signaling by EGF (161). However, we did not find any member of the ERK signaling cascade as a binding partner of IQGAP1 in human adipocytes. Instead, we found that IQGAP1 seems to be involved in insulin mediated interaction of caveolae with cytoskeletal elements (162). Meanwhile, β -arrestin has been shown to bind to Raf-1, MEK1/2 and ERK and restrict the activated ERK to the cytoplasm (163). We tested a minimal model for scaffolding in Paper I and found that restricting its availability for rephosphorylation by scaffolding could also explain the transient kinetics of ERK phosphorylation (Figure 10).

Feedbacks

A number of feedbacks both positive and negative have been described in the ERK signaling cascade. Some of the examples for negative feedback include phosphorylation and inhibition of MEK1/2 by ERK and phosphorylation of c-Raf by ERK that inhibits its interaction with Ras GTPase. On the other hand, examples of positive feedback include ERK phosphorylation of a dual specificity phosphatase DUSP6 that targets it for proteosomal degradation and enhance ERK signaling [reviewed in (164)]. We therefore tested whether a positive or negative feedback could explain ERK dynamics and found that a simple feedback model could not explain the dynamic data from human adipocytes. This however, does not rule out the possibility that a more complex model could explain the data.

Dual phosphorylation

Dual phosphorylation of ERK is another interesting feature for which different mechanisms have been proposed which include processive and distributive mechanisms (165, 166). In a processive mechanism, MEK binds to ERK and phosphorylates the two residues one by one before releasing it. Meanwhile, in a distributive mechanism, MEK binds to ERK and phosphorylates one residue, releases it, and then binds single phosphorylated ERK, and phosphorylates the second residue. We found that a simple processive model could simulate the observed dynamics in mature adipocytes (Figure 10).

Figure 10: Hypothesis testing. The three mechanisms; nuclear translocation, scaffolding and dual phosphorylation shared a common feature that could be translated into a common minimal model.

The three mechanisms that could explain the dynamics in human adipocytes, i.e. scaffolding, nuclear translocation and dual phosphorylation shared a common feature between them: a dephosphorylated pool of ERK that is temporarily unavailable for rephosphorylation (Figure 10). This is in line with sequestration

of ERK in nucleus and other subcellular organelles (144, 167), a finding that concords with what we observed in human adipocytes where a significant fraction of ERK was found to be localized in the nucleus. Thus a common minimal model encompassing the shared feature of sequestration of dephosphorylated ERK (Figure 10) was integrated into the insulin signaling model and was placed downstream of both IR and IRS1 (discussed later).

CROSS TALK BETWEEN ERK AND PKB-mTORC1 PATHWAY

The mitogenic signaling through the ERK pathway and the metabolic signaling through the PI3K-mTOR pathway are known to intersect each other at several nodes. Studies have demonstrated that these pathways regulate each other both positively and negatively, particularly during growth factor stimulation [reviewed in (168)]. Inhibition of the ERK pathway enhanced EGF-induced activation of PKB in breast cancer cell models (169). Similarly, treatment of HEK293 cells with PI3K inhibitor LY294002, enhanced insulin-like growth factor (IGF) induced phosphorylation of ERK by relieving the PKB-mediated inhibitory phosphorylation of Raf (170). Furthermore, in Rat2 fibroblasts PKB and ERK pathways have been shown to act synergistically to enhance mTORC1 signaling through phosphorylation of distinct sites on TSC2 (171). ERK has also been shown to promote mTORC1 activity by phosphorylating multiple residues on raptor (172), as well as regulate S6K2 activity in cardiomyocytes (173). Potent effect of growth factors on the ERK pathway and a comparatively small effect of insulin on ERK activation is possibly one of the reasons why insulin mediated ERK pathway is under-investigated.

In Paper I, we further address this question by examining the cross-talks between the insulin-regulated ERK pathway and the metabolic branch of insulin signaling. We found that inhibition of ERK reduced insulin-induced phosphorylation of ribosomal protein S6 at Ser^{235/236} by about 50%, demonstrating a significant crosstalk between ERK and mTORC1 for control of protein synthesis (Paper I). This possibly occurs via p90RSK that is a known downstream substrate of ERK (174) (Figure 11). Absence of any effect of ERK inhibition on insulin-induced S6K1 and IRS1 phosphorylations excluded the above discussed possibility of ERK-mediated activation of S6K or mTORC1 in human adipocytes (Paper I). In addition, EGF has been shown to induce phosphorylation of FOXO1 via ERK and

p38 MAPK, at sites distinct from the PKB phosphorylated sites in NIH3T3 cells that transiently expressed FOXO1 (175).

Interestingly, in Paper II we found that inhibition of ERK with MEK-specific inhibitor PD184352 inhibited phosphorylation of FOXO1-Ser²⁵⁶ by 35%. Thus, to the best of our knowledge, we show for the first time that phosphorylation of FOXO1 at Ser²⁵⁶ is mediated by both ERK and PKB in response to insulin in human adipocytes. This demonstrates another significant cross-talk between the insulin mediated ERK and PKB pathway for transcriptional control. Figure 11 indicates these newly identified cross-talks (black arrows) in the insulin signaling pathway in the human adipocytes.

Figure 11: Cross-talks in the insulin signaling network identified in our study (black arrows). Insulin-induced phosphorylation of ERK mediates the phosphorylation of FOXO1 at Ser²⁵⁶ and S6 at Ser^{235/236} in human adipocytes.

ERK SIGNALING IN DIABETES

Research findings on insulin-mediated ERK signaling in obesity and T2D have been very inconsistent. Results have been inconsistent not only between animal models and humans but also between different tissues. Impaired ERK phosphorylation has been reported in skeletal muscle of obese diabetic *ob/ob* mice (176) and obese Zucker rats (177), while increased ERK phosphorylation has been reported in liver of obese and diabetic *ob/ob* mice as compared to wild-type control mice (178). At the same time, there have been reports showing that insulin stimulation of ERK phosphorylation is unaffected in the skeletal muscle from obese and diabetic subjects (179, 180). Furthermore, only basal increase in ERK phosphorylation has been observed in human adipocytes (181) as well as in skeletal muscle from T2D subjects (182). Insulin-stimulated ERK phosphorylation is also reported to be increased in subcutaneous microvascular endothelial cells in T2D compared to healthy control subjects (183). Also, ERK activity has been shown to be increased in hypertrophic 3T3-L1 cells, adipose tissues of T2D mouse models as well as in rat L6 myotubes. Additionally, inhibition ERK activity improved insulin sensitivity and glucose tolerance in these cell types and animal models (184-186).

We have shown previously that exposure to short-term high calorie diet in lean individuals induced insulin resistance but enhanced ERK activation in the lean individuals (187). In contrast, in Paper I, we found that phosphorylation of ERK as well as its substrate Elk1 is impaired in obese individuals with T2D while the protein levels for ERK and Elk1 did not change between the normal and diabetic subjects. ERK has been suggested to be involved in mitotic clonal expansion during adipocyte differentiation (188). Short-term overeating and rapid weight gain, as in the case of the mentioned clinical study and animal models (diet-induced diabetes model), most likely involves increased adipogenesis, which could explain the enhanced ERK activation in these cases. However, obese diabetic individuals usually undergo a long-term gradual weight gain that probably affects the ERK signaling differently. Apart from reduced activation, we also show that ERK phosphorylation in adipocytes from diabetic subjects exhibits an increased EC_{50} of about 0.7 nM as compared to 0.2 nM in normal subjects. While there is a 10-fold increase in EC_{50} in diabetes for IRS1, there is no change in EC_{50} for IR (86). We have shown previously that phosphorylation of ERK and Elk1 occurs downstream of IRS1 (148), but ERK has also been shown to be activated by other adaptor proteins (discussed previously). Compared to ERK

phosphorylation, IRS1 displayed a dramatic increase in EC_{50} from 0.28 nM to 2.4 nM in diabetic adipocytes (86). Therefore, in the insulin signaling model, ERK was allowed to be activated by both IRS1 and IR via a second adapter protein, in order to capture the lack of loss of insulin sensitivity in ERK phosphorylation in the diabetic adipocytes.

FOXO1 IN HUMAN ADIPOCYTES

Forkhead box O (FOXO) family of transcription factors are evolutionarily conserved across species and comprise four isoforms namely FOXO1, FOXO3, FOXO4 and FOXO6. Of these, FOXO1 is the most abundantly expressed isoform in metabolic tissues i.e. adipose tissue, skeletal muscle, liver and β -cells. It is implicated in a diverse array of biological functions encompassing cell survival and cell metabolism, and has gained particular attention for their role in maintenance of cellular energy homeostasis [reviewed in (189)]. FOXO1 can be regulated by a variety of external stimuli like growth factors such as insulin or IGF-1, cytokines or oxidative stress. These stimuli tightly control FOXO1 activity through post-translational modifications such as phosphorylation, acetylation, ubiquitination and methylation. The post-translational modifications regulate the transcriptional activity of FOXO1 by altering its subcellular location, DNA binding ability and/or its protein levels [reviewed in (190)].

Figure 12: FOXO1 protein structure. All FOXO proteins consist of four domains forkhead DNA binding domain (Forkhead DBD), nuclear localization signal (NLS), nuclear export sequence (NES) and the C-terminal transactivation domain (TAD). Some important post-translation modifications and their corresponding kinases are shown. “P” denotes phosphorylation site and “A” denotes acetylation site. Refs 189 and 191 (boundaries of DBD varied between different papers)

FOXO1 can act both as a transcriptional activator or repressor depending on cell type, developmental stage and probably the associated cofactors. FOXO proteins comprise four domains; the highly conserved forkhead DNA binding domain, nuclear localization signal (NLS), nuclear export sequence and the C-terminal transactivation domain (190, 191) (Figure 12).

INSULIN REGULATION OF FOXO1 ACTIVITY

Insulin inhibits the transcriptional activity of FOXO1 through PKB-mediated phosphorylation of FOXO1 at Thr²⁴, Ser²⁵⁶ and Ser³¹⁹ (human sequence) (189) (Figure 13). The phosphorylation of these sites leads to nuclear exclusion of FOXO1 and hence inhibition of its transcriptional activity. Nuclear–cytoplasmic shuttling of FOXO1 has been reported to be regulated by the chaperone protein 14-3-3 which binds to phosphorylated FOXO1 and masks the NLS, hence preventing its entry in the nucleus (192).

Insulin-induced phosphorylation of FOXO1 in human adipocytes exhibited a rapid response ($t_{1/2}$ ~5min) and had a very low EC₅₀ of 0.02 nM for phosphorylation in response to insulin (Paper II). As discussed before, PKB activity is regulated by two phosphorylation sites Thr³⁰⁸ and Ser⁴⁷³. Previous studies show that FOXO1 phosphorylation depends on PKB phosphorylated at Ser⁴⁷³. Ablation of rictor or mLST8 in mouse embryo fibroblasts (MEFs) dramatically reduced the insulin-induced phosphorylation of FOXO3 but not the phosphorylation of TSC2 or GSK-3 β (124). Similarly, rictor-null fibroblasts exhibited an impaired phosphorylation of PKB Ser⁴⁷³ while the phosphorylation of S6K1 at Thr³⁸⁹ remained intact (193).

Homozygous deletion of SIN1, a component of mTORC2 completely abolished the insulin-stimulated phosphorylation of PKB at Ser⁴⁷³ in MEFs, which significantly reduced insulin-stimulated phosphorylation of FOXO1/3 but not the phosphorylation of GSK α/β and TSC2 (122). In line with this, we examined the regulation of FOXO1 in human adipocytes and showed that inhibition of both mTORC1 and mTORC2 by an mTOR inhibitor torin, eliminated insulin-induced phosphorylation of FOXO1 (Paper II). On the other hand, inhibition of mTORC1 by its specific inhibitor rapamycin did not affect the phosphorylation of either PKB at Ser⁴⁷³ or of FOXO1 at Ser²⁵⁶. This indicates that insulin-induced phosphorylation of FOXO1 at Ser²⁵⁶ is regulated by mTORC2-PKB Ser⁴⁷³

pathway and is largely independent of mTORC1, and consequently independent of the mTORC1-mediated positive feedback to IRS1 [Paper I, Paper II, (86)].

Figure 13: Insulin stimulated phosphorylation of FOXO1 is mediated by mTORC2 mediated phosphorylation of PKB at Ser⁴⁷³. Active PKB phosphorylates three conserved residues on FOXO1, Thr²⁴, Ser²⁵⁶ and Ser³¹⁹ (human sequence). Phosphorylation of FOXO1 by PKB leads to its nuclear exclusion and hence inhibition of its transcriptional activity.

REGULATION OF FOXO1 BY DIFFERENT POST-TRANSLATIONAL MODIFICATIONS OTHER THAN PHOSPHORYLATION

Apart from phosphorylation, FOXO1 activity can also be regulated by reversible acetylation catalyzed by histone acetyltransferases such as cAMP-response element-binding protein (CREB)-binding Protein (CBP) and p300 under conditions of oxidative stress, and can be deacetylated by NAD-dependent histone deacetylases such as sirtuins 1, 2 or 3 (SIRT1/2/3) and histone deacetylase

2 (HDAC2) (194). Acetylation of transcription factors is generally considered as an “activating” modification. However, acetylation of FOXO1 at Lys²⁴⁵, Lys²⁴⁸, Lys²⁶⁵ (human sequence), which lies in the DNA binding forkhead domain (Figure 12), has been shown to attenuate its transcriptional activity by impairing its DNA binding ability, while deacetylation increases its transcriptional activity (194).

Figure 14: In addition to phosphorylation, acetylation status of FOXO1 also regulates its transcriptional activity. Phosphorylated FOXO1 can also be ubiquitinated in the cytoplasm that leads to its proteasomal degradation. P-phosphorylation, Ac- acetylation and Ub-ubiquitination.

For example, knockdown of SIRT2 decreased the association, while overexpression of SIRT2 increased the association between FOXO3a and p27 promoter in NIH3T3 cells as shown by chromatin immunoprecipitation (195).

In addition to regulating the DNA binding ability, acetylation of FOXO1 can also promote phosphorylation of FOXO1 at Ser²⁵⁶ and its subsequent nuclear exclusion. It has been suggested that acetylation and phosphorylation cooperatively inhibit the transcriptional activity of FOXO1, where acetylation of

FOXO1 destabilizes the FOXO1-DNA association and facilitates PKB-mediated phosphorylation of FOXO1 at Ser²⁵⁶ (196) (Figure 14). FOXO1 acetylation has also been reported to inhibit adipogenesis and the interplay between acetylation and phosphorylation has also been observed during the process of adipocyte differentiation. For example, reduced SIRT2 expression increased adipogenesis, which is accompanied by increase in FOXO1 acetylation (197).

Ubiquitination of FOXO1 adds another layer of regulation to FOXO1 activity by regulating its protein levels via the ubiquitin-proteasome system. Thus, apart from triggering nuclear exportation, phosphorylation of FOXO1 by PKB can also induce degradation of FOXO1. The half-life of FOXO1 following insulin stimulation has been shown to be 4-6 hours in HepG2 cells (198), whereas platelet-derived growth factor (PDGF) stimulation induces rapid degradation (~15 min) of FOXO1 in chicken embryo fibroblasts (199). This mechanism, however, does not seem to operate in human adipocytes as we did not observe any rapid insulin-induced degradation of FOXO1, as observed after 90 min time course with insulin (Paper II). Our findings also show that inhibition of mTORC2 by long-term rapamycin treatment that has been observed in some cell types does not occur in human adipocytes [(141), Paper II].

Both acetylation and ubiquitination occur on lysine residues and hence these post translational modifications could also inversely affect each other. For example, under conditions of oxidative stress, FOXO1 acetylation has been shown to prevent ubiquitin-mediated proteasomal degradation of FOXO1 in pancreatic β -cells (200).

FOXO1 IN TYPE 2 DIABETES

Owing to its role in hepatic glucose production and inhibition of adipogenesis, FOXO1 is generally considered to be “pro-diabetic”. This is observed in number of animal studies, where disruption of FOXO1 improved glucose homeostasis and insulin sensitivity. For example, genetic deletion of a single FOXO1 allele restored insulin sensitivity in insulin receptor deficient mice by reducing hepatic gluconeogenesis, increasing β -cell compensation and increasing the expression of insulin sensitizing genes in adipose tissue. In contrast, transgenic mice expressing gain-of-function mutant for FOXO1 in liver and pancreas became diabetic due to impaired β -cell compensation and increased hepatic glucose production (201).

Furthermore, FOXO1 haplosufficiency protected the mice from developing insulin resistance and diabetes in diet-induced diabetes mice models (202). Also, hepatic expression of dominant negative FOXO1 in a leptin receptor-deficient diabetes mouse model (*db/db*) improved the fasting blood glucose levels through regulation of expression levels of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (203). Compared to matched controls, an increased nuclear expression has been reported in liver of *db/db* mice (203), but on the other hand, FOXO1 expression has been shown to be decreased in adipose tissue of *db/db* mice (204). Though a lot has been investigated in animal models, little is known about how FOXO1 is affected in diabetes in humans. Table 1 summarizes the phenotypic alterations in animal models as a result of changes in FOXO1 expression.

In Paper II, we investigated the insulin regulation of FOXO1 in both normal and diabetic individuals. We reported that the abundance of FOXO1 is reduced by 45% in mature adipocytes from diabetic subjects as compared to the lean controls. Both control and diabetic subjects exhibited similar response time, but the level of phosphorylation was lower in diabetes. Both the reduction in phosphorylation and acetylation of FOXO1 in adipocytes from diabetic individuals could be attributed to the reduced protein levels of FOXO1 in diabetes. Likewise, insulin sensitivity for the phosphorylation of FOXO1-Ser²⁵⁶ was not different between the diabetics and control subjects, with an EC₅₀ of about 0.02 nM.

We also showed that FOXO1 lies downstream of PKB-Ser⁴⁷³ which has been shown previously to be unaffected in T2D [Paper II, (86)] and hence would explain why the regulation FOXO1 by insulin is unperturbed in diabetic adipocytes. While these findings show that insulin regulation of FOXO1 is not impaired in diabetes, the reduced levels of FOXO1 in diabetic adipocytes would result in an overall lower FOXO1 activity in the cells.

As mentioned before, reduced positive feedback from mTORC1 to IRS1 is the dominant mechanism that induces insulin resistance in human adipocytes, therefore we also examined the effect of long-term inhibition of mTORC1 (48 h) with rapamycin on insulin signaling intermediaries. Prolonged inhibition of mTORC1 reduced the protein levels of FOXO1, but other proteins like IR, S6, AS160 and GLUT4, which are reduced in T2D, remained unaffected (Paper II). However, changes at post-translational level require time to manifest and therefore changes secondary to FOXO1 reduction would be difficult to detect in a period of 48 hours.

Table 1: Effects of altered FOXO1 expression in various mouse models.

Genetic alterations	Phenotypes	Refs
FOXO1 haplosufficiency in diabetic phenotype (InsR^{+/-}, Irs2^{-/-})	Improved whole body insulin sensitivity with decreased hepatic glucose production, increased pancreatic β cell compensation, and increased expression of insulin-sensitizing genes in adipose tissue	201, 218
FOXO1 haplosufficiency in high fat fed mice	Protected from diet-induced insulin resistance and diabetes	202
LIVER		
Transgenic expression of constitutively active FOXO1 in liver	Impaired glucose tolerance, mild hyperinsulinemia, adult onset diabetes	201
Adenoviral expression of constitutively active FOXO1 in liver	Reduced plasma insulin, glucose, and TG levels in the fed state, increased TG synthesis, decreased FFA oxidation	215
Liver specific knockout of FOXO1/3/4	Improved whole-body insulin sensitivity, reduced fasting and fed blood glucose levels, increased serum triglyceride, cholesterol and VLDL secretion; hepatosteatosis	208, 213, 214
Liver specific FOXO1 knockout in mouse models with hepatic insulin resistance	Improved whole-body and hepatic glucose metabolism, improved plasma insulin levels, improved mitochondrial oxidative metabolism and biogenesis	203, 205-207
Adenoviral expression of dominant negative FOXO1 in liver of db/db mice (leptin-receptor deficient diabetes model)	Reduced hepatic glucose production due to reduced gluconeogenic gene expression, improved fasting blood glucose levels	203
β-CELLS		
Transgenic expression of constitutively active FOXO1 in β-cells	Impaired glucose and insulin tolerance, decreased β -cells proliferation leading to β -cells failure and early onset diabetes	201

Genetic alterations	Phenotypes	Refs
SKELETAL MUSCLE		
Muscle-specific deletion of FOXO1	Fiber type switch from fast to slow-twitch muscle, impaired glucose tolerance, muscle atrophy due to increased protein degradation	221, 222
ADIPOSE TISSUE		
Adipose tissue-specific transgenic expression of dominant negative FOXO1	Increased whole body insulin sensitivity, improved glucose tolerance, increased number of small-sized adipocytes, increased adiponectin and GLUT4 and reduced pro-inflammatory adipokines, increased energy expenditure in brown adipose tissue	226

Being one of the major transcription factors regulated by insulin, FOXO1 is likely to be involved in transcription of important insulin signaling intermediaries as has been shown in other tissues (discussed in the next section). Thus, examining the effects of reduced FOXO1 abundance would not only give insight into the function of FOXO1 in adipocytes but also identify its role in development of T2D, which was thus the motivation for Paper III.

Intriguingly, we found that inhibition of FOXO1 in adipocytes with a dominant negative FOXO1 (FOXO1-DN) mutant impaired insulin signaling in a manner that was strikingly similar to insulin signaling in T2D. The similarity was emulated in all parts of the insulin signaling network namely control of ERK, phosphorylation of PKB at Ser⁴⁷³, and mTORC1 including S6K1 phosphorylation and the positive feedback signal to IRS1 at Ser³⁰⁷ (Figure 15). Additionally, protein levels of IR, S6, mTOR and raptor were also reduced following FOXO1 inhibition in mature adipocytes. Reduced mTOR and raptor levels indicated reduced mTORC1 activity and consequently reduced mTORC1-mediated feedback. Examining further, we also found that inhibition of FOXO1 for 72 hours in differentiated human pre-adipocytes resulted in reduction of GLUT4 as well (Paper III).

We used the comprehensive insulin signaling model (next section) to understand the mechanism that impaired insulin signaling in the FOXO1-inhibited adipocytes. The reduced protein levels were introduced in the model, which impaired the signaling but not to the same extent as in the experiment. We therefore tested different levels of attenuation of mTORC1 and found that

together with reduced protein levels, attenuation of mTORC1 activity to 25% gave the best agreement with the FOXO1-DN experimental data.

Figure 15: Similarity between the diabetic state (red dots with error bars) in human adipocytes and insulin signaling following transfection of human adipocytes with FOXO1-DN for 48hrs (green lines). Blue dots with error bars represent non-diabetic subjects while blue lines represent GFP transfected adipocytes. Data are from Paper III (FOXO1-DN data) and 86 (diabetes data).

It should be noted that the extent of reduction of mTORC1 activity for agreement of model with the data was entirely consistent with the experimentally observed reduction of S6K1 phosphorylation to 15% of controls, which is a direct read-out of mTORC1 activity (Figure 15). Interestingly, this reduction is also comparable to the previously reported reduction of the feedback to 15% of the control adipocytes in diabetes (86). Thus, inhibition of FOXO1 not only mimicked the changes in some of the protein levels in T2D, but also the reduced mTORC1 activity similarly as observed in the diabetic adipocytes. This is a striking finding as it is contrary to the prevalent notion that FOXO1 activity is detrimental to insulin signaling in the diabetic state. Our findings in fact suggest that FOXO1 activity is important to maintain normal insulin signaling in mature human adipocytes.

ROLE OF FOXO1 IN NON-ADIPOSE TISSUES

As discussed previously, FOXO1 activity is generally negatively associated with diabetes. Lack of inhibition of FOXO1 activity due to insulin resistance is considered to be the main culprit behind increased glucose production in diabetes. In liver, which is a primary organ for glucose production, insulin inhibition of FOXO1 reduces the expression of PEPCK and G6Pase and hence reduces hepatic glucose release (Figure 16). A number of studies in different animal models with hepatic insulin resistance have shown that loss of FOXO1 improves the blood glucose and insulin concentrations and mitochondrial metabolism (203, 205-207). However, disruption of FOXO1 in liver of mice with intact insulin receptor function requires loss of multiple FOXO isoforms to observe a significant decrease in plasma glucose levels (208, 209), whereas deletion of FOXO1 in liver of mice lacking hepatic insulin receptors has a dramatic effect on glucose homeostasis (206). This thus indicates that despite compensatory mechanisms and the involvement of other isoforms of FOXO in maintenance of glucose homeostasis in liver, FOXO1 is a major regulator of glucose homeostasis in the insulin resistant state.

One of the interesting features of T2D is that insulin resistance co-exists with other aspects that display increased insulin sensitivity such as increased lipid synthesis (210) and reduced fatty acid oxidation (211). This can be explained by the other major function of FOXO1 in liver, which is regulation of lipid

metabolism. FOXO1 is involved in regulation of hepatic production and secretion of very low density lipoproteins (VLDL) and can regulate a number of proteins involved in lipid synthesis, such as sterol regulatory element-binding protein-1c (SREBP-1c), acetyl-CoA carboxylase- α (ACC), and fatty acid synthase (FAS), and in fatty acid oxidation like PPAR α (212) (Figure 16). In line with this, ablation of hepatic FOXO1 in mice, contributed to the lipid abnormalities observed in the insulin resistant state and exhibited increased VLDL secretion, increased plasma levels of triglycerides, cholesterol and FFAs (208, 213, 214). In contrast, acute overexpression of constitutively nuclear FOXO1 in mouse liver increased hepatic triglyceride content, however, these mice exhibited improved plasma glucose, insulin and triglyceride levels in the fed state (215).

In pancreatic β -cells, FOXO1 can inhibit β -cell replication by regulating expression of cell cycle inhibitors like p16 and p27, and of transcription factors like PDX1. FOXO1 has also been suggested to cooperate with notch signaling to repress NEUROG3 transcription and hence inhibit β -cell neogenesis (216, 217) (Figure 16). In IRS2 knockout mice, which develop T2D due to β -cell failure, FOXO1 haplosufficiency was sufficient to restore β -cell proliferation (218). Another interesting finding was that inhibition of FOXO1 in gut endocrine progenitor cells generated glucose responsive insulin producing cells (219). These studies indicate an important role of FOXO1 in β -cell compensatory response in the insulin resistant state. In addition, FOXO1 activity is required under hyperglycemia-induced oxidative stress for protection against β -cell failure through induction of insulin-gene transcription factors NeuroD and MafA (200). Also, overexpression of FOXO1 enhanced β -cell proliferation under conditions of low nutrition (220). Thus, through the effects on β -cell growth and physiology, FOXO1 might play a very important role in the transition from insulin resistance to T2D.

FOXO1 also plays a critical role in maintaining skeletal muscle function by regulating the muscle mass or fiber type expression (Figure 16). Transgenic mice overexpressing FOXO1 in skeletal muscle exhibited impaired glycemic control due to muscle atrophy and hence exhibited reduced muscle function (221, 222). FOXO1 is also known to induce expression of pyruvate dehydrogenase kinase 4 (PDK4) in muscle during fasting, to inhibit glucose oxidation and induce expression of lipoprotein lipase (LPL) and CD36 that promote fatty acid uptake and oxidation (223-225) (Figure 16). FOXO1 thus appears to control the substrate

selection in muscle, depending upon the nutritional status of the body, to switch between glucose oxidation and fatty acid oxidation.

Figure 16: Possible roles of FOXO1 in muscle, liver and pancreas.

ROLE OF FOXO1 IN ADIPOSE TISSUE

In adipose tissue, FOXO1 is particularly known for its role as an inhibitor of adipogenesis. Inhibition of FOXO1 with dominant negative FOXO1 has been reported to rescue adipocyte differentiation of embryonic fibroblasts from insulin receptor deficient mice (202). It is known to control the process of adipogenesis in mice through suppression of both expression and activity of PPAR γ and C/EBP α , as well as cell cycle proteins such as p21 and p27 (202). Transgenic

mice expressing dominant negative FOXO1 in the adipose tissue have improved insulin sensitivity which is attributed to increased adipogenesis and hence increased number of small insulin-sensitive adipocytes (226). There have been many studies in animal models that show that FOXO1 inhibits the transcriptional activity of PPAR γ (227-229), although positive regulation of PPAR γ by FOXO1 has also been reported in 3T3-L1 cells (230). However, from expressing DN-FOXO1, we found that in mature human adipocytes PPAR γ may be positively regulated by FOXO1. Since our finding was contrary to most of the observations in animal models, we further examined expression of PPAR γ in differentiated human pre-adipocytes following inhibition of FOXO1 and found a similar result (Paper III).

PPAR γ is responsible for differentiation of pre-adipocytes and hence formation of new adipocytes. PPAR γ is known to improve whole body insulin sensitivity in both animal models and clinical studies [reviewed in (231)]. Increase in the number of small adipocytes leads to an increase in the lipid storing capacity of the adipose tissue which maintains insulin sensitivity of the adipose tissue by increasing adipogenesis and avoiding ectopic fat deposition. The inability of the diabetic adipocytes to store fatty acids indicates that PPAR γ expression/activity is impaired, which is in line with the reduced FOXO1 expression in adipocytes from T2D patients (Paper II). PPAR γ has also been shown to be involved in transcription of key intermediaries of lipid metabolism in the adipose tissue such as LPL, adipocyte Protein 2 (aP2), PEPCCK and acetyl coA-synthetase (ACS) (231, 232). Thus, reduced FOXO1 abundance in the diabetic adipocytes would consequently result in dysfunctional adipocytes not only by affecting the gene expression of its direct targets but also targets downstream of PPAR γ . In line with this, it is really interesting that adipocyte-specific activation of PPAR γ was sufficient to increase the whole body insulin sensitivity in mice (233).

A well-known target of PPAR γ that has been reported in rodent models is the glucose transporter protein GLUT4. However, the direction of this particular regulation remains controversial as PPAR γ has been reported to be both a positive and a negative regulator of GLUT4 expression in mice and 3T3-L1 adipocytes (229, 234, 235). We detected downregulation of GLUT4 mRNA after 72h in differentiated human pre-adipocytes, following inhibition of FOXO1 but not after 48 hours in mature adipocytes, which indicates that the downregulation of GLUT4 was secondary to the reduction of PPAR γ . This was further supported by the reduction in the protein levels for GLUT4 in the differentiated human pre-

adipocytes after 72 h with the dominant negative mutant of FOXO1 (Paper III). This suggests that reduction in FOXO1 levels most likely contributes to the GLUT4 reduction in the diabetic adipocytes, which is responsible for the reduced maximal glucose uptake by adipocytes in T2D.

Consistent with the involvement of FOXO1 in lipid metabolism in liver, we found that FOXO1 may positively regulate adipose triglyceride lipase (ATGL) in human adipocytes (Paper III). ATGL and hormone sensitive lipase (HSL) are key enzymes involved in lipid catabolism. In adipocytes, FOXO1 could thus promote breakdown of lipids by regulating the expression of ATGL. This is in agreement with a previous study in 3T3-L1 adipocytes where FOXO1 has been shown to regulate the expression of ATGL (236). We also found that inhibition of FOXO1 with the dominant negative mutant reduced the mRNA expression of PDK4 and CD36 in the adipocytes (Paper III), which is consistent with observations in murine C2C12 muscle cell line and human liver cell line HepG2 (224, 237). PDK4 has been shown to regulate fatty acid esterification via glyceroneogenesis in the adipose tissue (238), while CD36 is involved in fatty acid transport and uptake. This indicates that by regulating PDK4 and CD36 expression, FOXO1 could regulate the release of fatty acids from the adipocytes, a process that is dysregulated in T2D. Thus, regulation of ATGL, PDK4 and CD36 in adipocytes by FOXO1, together with its role in liver and muscle strengthens the role of FOXO1 in maintaining whole body lipid homeostasis.

Adipose tissue is known to secrete a plethora of adipokines such as TNF α , adiponectin and leptin. These adipocyte derived factors play an important role in tissue cross-talk and maintenance of whole-body metabolic homeostasis. Adiponectin has gained the attention since it is an insulin sensitizing hormone and circulating adiponectin levels appears to be one of the strongest predictors of metabolic syndrome that includes insulin resistance, T2D, cardiovascular events, and hypertension (239-241). FOXO1 has been reported to form a complex with C/EBP α at the mouse adiponectin promoter to enhance adiponectin gene expression (242). Furthermore, free fatty acid exposure has been shown to reduce FOXO1 expression in 3T3-L1 adipocytes with a concomitant increase in pro-inflammatory cytokines and decrease in adiponectin expression (204). Consistent with these studies, we found that FOXO1 inhibition reduced adiponectin expression in both primary human adipocytes as well as differentiated human pre-adipocytes, further indicating a protective role of FOXO1 in adipocytes (Paper

III). We also observed a tendency towards increase in the expression of pro-inflammatory cytokine TNF α , following inhibition of FOXO1 (Paper III).

Cell cycle control and cell fate determination has been widely studied in context of adipogenesis. Adipocyte differentiation involves a proliferative step called clonal expansion that is followed by cell differentiation. This process requires an intricate crosstalk between cell cycle regulation and developmental and differentiation pathways. Induction of adipocyte differentiation is accompanied by induction of cell cycle inhibitors such as p27, p21 and pRb to initiate the cell cycle arrest (202), however little is known about the regulation of these proteins in mature adipocytes.

Figure 17: Transcriptional targets of FOXO1 in adipose tissue identified in our study.

Our findings indicate that FOXO1 inhibits p21 expression in mature human adipocytes (Paper III). Expression of p21 has been shown to transiently increase during differentiation and declines when the cells are terminally differentiated (243). Thus, it is probable that FOXO1 transiently modulates p21 expression during the process of adipocyte differentiation and switches it off when the cells are terminally differentiated. This is supported by a recent study that showed very dynamic kinetics of FOXO1 expression and activation during adipocyte differentiation, following a series of sigmoidal curves (230). This also suggests that experiments with stable overexpression or inhibition of FOXO1 during the

differentiation process should be considered carefully for conclusions. We also found that FOXO1 may regulate the expression of Rbl2 in mature adipocytes (Paper III) which has been suggested to trigger and maintain cellular senescence (244). Thus, by regulating expression of p21 and Rbl2, FOXO1 may be involved in maintaining the terminally differentiated state of the adipocytes. Figure 17 summarizes the different transcriptional targets of FOXO1 in mature adipocytes.

COMPREHENSIVE MATHEMATICAL MODEL OF THE INSULIN SIGNALING NETWORK

Cellular signaling networks such as insulin signaling are remarkably complex with multiple inputs, interactions, cross-talks and feedbacks. Complexity of the signaling pathways is thus one of the biggest challenges in our efforts to delineate the signaling networks and to understand molecular pathogenesis of diseases. It is not possible for a human mind to take into account all the potential variables in the network. It is unsure how a small sub-module of the network would perform when embedded into the entire network or vice versa. Thus, the conclusions derived through experimental approaches need to be assessed when applied to complex biological systems as a whole. It is therefore important to adopt new approaches and tools and it is here that computational methods and mathematical modeling also known as ‘systems biology’ comes into picture.

Examining how our results affected the entire insulin signaling network was important to evaluate our findings in a physiological context. Our findings, together with the comprehensive insulin signaling model presented in this thesis have taken our understanding of pathogenesis of insulin resistance in adipocytes in T2D to the next level. Paper II thus presents the comprehensive insulin signaling model for the human adipocytes that describes insulin control via its various branches to the control of glucose uptake, autophagy, protein synthesis and transcriptional regulation via ERK and FOXO1 (Figure 18).

In order to analyze our data at the network level, we integrated our data from Paper I and II into the insulin signaling model presented in Brännmark et al. (86) (Figure 18). Insulin control of ERK was integrated as the minimal model derived from the hypothesis testing to understand ERK phosphorylation dynamics. ERK was allowed to be activated by both IR and IRS1 (discussed previously). Insulin control for FOXO1 was added downstream of PKB-Ser⁴⁷³, with limited contribution from PKB-Thr³⁰⁸. The model also includes the newly identified cross-talks from ERK to S6 and FOXO1. The comprehensive model reproduced

the signaling data throughout the network in human adipocytes (Figure 19, blue lines).

Figure 18: Comprehensive mathematical model for insulin resistance in T2D. Given is the structure of the complete mathematical model for insulin signaling and insulin resistance in human adipocytes including the ERK and the FOXO1 module. To simulate the diabetic state in the model, the diabetes parameters (shown in box) were used. The main diabetes parameter (15% feedback) was fitted to the data. All the other diabetes parameters were based on measured protein levels in Paper I & II, except for IR (187) and GLUT4 (256) .

To simulate the diabetic state, differences between the normal and diabetic states (diabetes parameters) were introduced into the model: i) Previously reported reduced abundances of IR and GLUT4 together with newly identified reductions in protein levels of FOXO1, AS160 and S6; ii) Reduced feedback from mTORC1 to phosphorylation of IRS1 Ser³⁰⁷. The reduction of the feedback was not directly measured, but estimated based on all available data. When these differences were introduced, and all other parameters were kept from the normal simulation, the model replicated the diabetic state in the human adipocytes (Figure 19, red lines).

Figure 19: Model simulations were in agreement with normal and diabetes data. Shown are model simulations (lines) using the mathematical model in Fig. 18 for insulin signaling, normally (blue) and in T2D (red), and comparisons with the corresponding experimental data [dots and error bars (S.E.)] for the indicated signaling intermediaries. Normal simulations were scaled with normalization constants to the experimental data (blue). The T2D simulations were obtained by changing the diabetes parameters as shown in Fig. 18. (Data from 85, 86, Paper I and II). a.u., arbitrary units.

One of the interesting things that can be done with the model is to evaluate the contribution or impact of individual diabetes parameters (Figure 20).

Figure 20: The impact of the individual diabetes parameters. Shown are heat maps of the impact of the individual diabetes parameters on the measured model variables. Light color indicates a small contribution, and dark color indicates a large contribution. The mTORC1 feedback contributes to the reduction in the steady-state level of phosphorylation and to the increased EC_{50} all over the insulin-signaling network. The reduction of IR levels also has slight effects network-wide, whereas all other diabetes parameters reflect peripheral protein levels, the reductions of which only have local effects. **A**, the contribution to the reduction in the steady-state level of phosphorylation for the diabetes parameters is calculated as the reduction from normal in the insulin-stimulated steady state for a single diabetes parameter (as indicated) divided by the reduction from normal in the diabetes simulation with all diabetes parameters. **B**, the contribution to the increase in EC_{50} for the diabetes parameters is calculated as the increase above normal in EC_{50} for a single diabetes parameter (as indicated) divided by the increase above normal in the diabetes simulation with all diabetes parameters.

Reduction in AS160, GLUT4 and S6 had only local effects, while reduction in IR affected its insulin-induced autophosphorylation and also signaling via AS160 and ERK. Attenuation of feedback from mTORC1 to IRS1, on the other hand contributed to the reduction in the steady state responses as well as the insulin

sensitivities of all insulin signaling intermediaries (except PKB-Ser⁴⁷³ and FOXO1) in the network. We have experimentally verified this mTORC1-dependent induction of network-wide insulin resistance using rapamycin, an mTORC1-specific inhibitor. Rapamycin inhibition resulted in reduced glucose uptake, protein synthesis and ERK signaling, and increased autophagy [(86, 87), Paper I].

Furthermore no effect of rapamycin was observed for insulin-induced phosphorylation of FOXO1, which was consistent with the predictions from the model simulations for mTORC1 inhibition. There have been contradictory findings for the effect of rapamycin on phosphorylation of PKB and ERK. Insulin-induced phosphorylation of PKB at Ser⁴⁷³ and Thr³⁰⁸ has been reported to increase in differentiated human pre-adipocytes in presence of rapamycin (245). We did not observe any effect of rapamycin on PKB Ser⁴⁷³ in our experiments [(86), Paper II]. Also, both short-term and long-term treatment with rapamycin in breast cancer cell line MCF-7 or murine macrophages has been shown to enhance ERK phosphorylation (246-248).

Table 2. Effect of rapamycin on insulin stimulated protein phosphorylation in human adipocytes. Increase in the measured protein phosphorylation is denoted by $\uparrow$, decrease is denoted by $\downarrow$, and no effect is denoted by $\leftrightarrow$

Measured Protein phosphorylation	Effect of rapamycin	Refs.
IR-Tyr	$\downarrow$	105
IRS1-Tyr	$\downarrow \leftrightarrow$	99, 105
IRS1-Ser ³⁰⁷	$\downarrow$	86, 99, 105
PKB-Thr ³⁰⁸	$\uparrow \leftrightarrow$	105, 245
AS160-Thr ⁶⁴²	$\downarrow$	105
PKB-Ser ⁴⁷³	$\downarrow \uparrow \leftrightarrow$	86, 105, 245
FOXO1-Ser ²⁵⁶	$\leftrightarrow$	Paper II
S6K1-Thr ³⁸⁹	$\downarrow$	Paper I
S6-Ser ^{235/236}	$\downarrow$	86
ERK1/2-Tyr ²⁰² /Thr ²⁰⁴	$\downarrow$	Paper I

On the other hand, in pancreatic cell line no effect of rapamycin was observed on insulin-stimulated phosphorylation of ERK (249). In human adipocytes, however, insulin-induced ERK phosphorylation was found to be reduced in the presence of rapamycin (Paper I). Table 2 enlists the effects of rapamycin on various insulin signaling intermediaries in human adipocytes and with few exceptions as discussed above, they were mostly in agreement with our observations.

MECHANISMS OF INSULIN RESISTANCE IN TYPE 2 DIABETES IN ADIPOCYTES

Transition of an individual from healthy to insulin resistant diabetic state involves changes in insulin signaling in adipocytes. These defects in signaling occur in different parts of the insulin signaling network. However, lack of data from physiologically relevant cells and lack of consistency of experimental conditions, combined with individual variation and complexity of signaling networks, have resulted in fragmented understanding of the network. Therefore, we have made an effort in this direction and through the work presented in this thesis, present a comprehensive understanding of insulin signaling in adipocytes through its various branches namely PKB-mTORC1, PKB-FOXO1 and ERK pathways, both normally and in T2D.

There seems to be an increasing consensus about origin of insulin resistance in the expanding adipose tissue. Insulin resistance can be detected in the adipocytes prior to diagnosis of T2D (250). Consistent with this idea, alterations in insulin signaling in adipocytes has been shown to improve metabolism systemically. For example, prolonging insulin action selectively in mature adipocytes in transgenic mice with adipocyte-specific deletion of PTEN (a phosphatase involved in turning off insulin signaling) improved systemic metabolic profile with enhanced whole body insulin sensitivity, improved glucose tolerance, increased adiponectin levels and reduced adipose tissue inflammation (251). Mice lacking GLUT4 specifically in adipose tissue develop insulin resistance in muscle and liver although the GLUT4 expression remained unchanged in muscle (252). Furthermore, transgenic mice with selective activation of PPAR γ in mature adipocytes exhibited increased insulin sensitivity and were protected against diet-induced insulin resistance (253). In co-culture studies, selective activation of PPAR γ in 3T3-L1 adipocytes suppressed pro-inflammatory cytokine production in macrophages (253). In spite of the importance of adipocytes in maintaining systemic insulin sensitivity, very little has been known about how insulin resistance develops in adipocytes.

Through a series of publications, we have unraveled the mechanisms that introduce the changes in insulin signaling in T2D. We have thus found a number of differences in insulin signaling between normal and diabetic adipocytes. These differences were observed in several insulin signaling proteins as i) reduced maximal phosphorylation in response to insulin at steady state; and ii) reduced insulin sensitivity (EC_{50}) (the concentration of insulin required for half maximal response) (Figure 19). Table 3 summarizes the changes that occur in various insulin signaling intermediaries in T2D.

Table 3: Summary of insulin responses and protein abundances of different insulin signaling intermediaries in the network. Steady state levels of phosphorylation and glucose uptake, determined at maximal insulin concentration, are presented as fold increase over non-diabetic controls at basal (without insulin) conditions. Contr: non-diabetic controls, nd: not determined, no-ss: no steady state, ns: not significantly different.

	Sensitivity EC_{50}		Response time $t_{1/2}$		Steady state level		Protein level	Refs.
	contr	T2D	contr	T2D	contr	T2D		
	<i>nM insulin</i>		<i>min</i>		<i>Fold over basal of control</i>		<i>% of control</i>	
IR-Tyr (P)	1.0	1.0	1.0	nd	4.3	1.5	55	86, 187
IRS1-Tyr (P)	0.3	2.0	1.0	nd	2.6	1.0	ns	99,86,187
IRS1-P-Ser ³⁰⁷	0.2	0.9	0.7	2.0	4.4	1.9		86
PKB-P-Thr ³⁰⁸	0.3	0.4	nd	nd	5.0	5.0	ns	86, Paper II
PKB-P-Ser ⁴⁷³	0.1	0.4	1.0	1.0	5.0	5.0		86,
AS160-P-Thr ⁶⁴²	0.02	0.2	1.5	2.0	2.9	1.3	45	86, Paper II
Glucose transport	0.04	0.2	nd	nd	2.1	1.0	50	86
S6K1-P-Thr ³⁸⁹	nd	nd	7.5	7.5	4.1	2.6	ns	86, 87
S6-P-Ser ^{235/236}	0.3	nd	15		4.3	2.5	48	86, Paper II
ERK1/2-P-Thr ²⁰² /Tyr ²⁰⁴	0.2	0.7	2.5	2.5	no-ss	no-ss	ns	Paper I
Elk-P-Ser ³⁸³	nd	nd	5		no-ss	no-ss	ns	Paper I
FOXO1-P-Ser ²⁵⁶	0.02	0.02	5	5	1.7	1.1	55	Paper II
mTOR							ns	Paper II
GLUT4							50	256

The change in insulin sensitivity seems to emanate from IRS1 since the insulin receptor does not display any change in insulin sensitivity (86). The reduced

insulin sensitivity of IRS1 propagates to a large part of the signaling network including the ERK branch of insulin signaling. In contrast, the mTORC2 branch that mediates the phosphorylation of PKB at Ser⁴⁷³ and of FOXO1 is not impaired in T2D as is evident from the insulin-stimulated time course and dose response curves for these proteins. Phosphorylation of PKB in response to insulin in adipocytes from diabetic subjects has been a bit puzzling. For example, phosphorylation of PKB at Thr³⁰⁸ does not display reduced sensitivity, although the absolute level of phosphorylation at Thr³⁰⁸ is reduced in T2D. On the other hand, phosphorylation at Ser⁴⁷³ tends to be higher in T2D. There have been reports of a negative feedback from mTORC1 to mTORC2 (254, 255), which could result in the increase, observed in phosphorylation of PKB at Ser⁴⁷³. Indeed, inhibition of mTORC1 prior to insulin stimulation resulted in a slight increase in the phosphorylation at Ser⁴⁷³, though the increase was not significant (86). Inability of the model to exactly replicate some of the experimental data indicates the presence of cross-talks and feedbacks that haven't yet been identified.

One of the fundamental changes that occur in the diabetic adipocytes is attenuation of mTORC1 activity, which is evident in the form of reduced feedback from mTORC1 to phosphorylation of IRS1 at Ser³⁰⁷, reduced phosphorylation of S6K1, reduced ribosome synthesis, increased autophagy and impaired mitochondrial function (86, 87). The reduced positive feedback from mTORC1 to IRS1 explains insulin resistance in major part of the network, including the ERK pathway (Figure 20). It is interesting that this feedback is attenuated in patients with T2D, and stimulation of this feedback with okadaic acid (a serine phosphatase inhibitor) rescued the diabetic cells from insulin resistance (99). Experimental inhibition of mTORC1 with rapamycin reduced insulin-mediated increase in glucose uptake, protein synthesis and ERK signaling and relieved insulin-mediated inhibition of autophagy. Also, treatment of adipocytes with rapamycin resulted in autophagy-mediated reduction in the number of mitochondria (86, 87). And similar to diabetes, no effect of rapamycin was observed on insulin-induced phosphorylation of FOXO1 which is consistent with PKB-Ser⁴⁷³ being unperturbed in diabetes.

Another major difference that we have found between normal and diabetic adipocytes was the reduced expression level of several insulin signaling intermediaries, which included IR, GLUT4, S6, AS160 and FOXO1 [(187, 256), Paper II]. GLUT4 protein levels have been shown to be reduced in numerous studies in adipocytes as well as muscle in T2D (181, 187, 256, 257). However,

there are conflicting reports for expression of proteins like IR and IRS1 in T2D which could be due to tissue-specific differences, population-specific differences, and can also depend on experimental conditions and handling of cells. For example, IRS1 has been reported to be reduced in adipocytes from individuals with T2D (181, 258). However, we have not found any difference in expression of IRS1 between adipocytes from normal and diabetic individuals (99, 187). Similarly, we have observed a reduction in IR expression in adipocytes in T2D to 55% of the normal protein levels (187), whereas another study has reported no change in IR protein levels between normal and T2D (258). Consistent with reduced abundance of FOXO1 in human adipocytes, FOXO1 target genes were also downregulated in muscle from obese diabetic subjects (259). Furthermore, reduced levels of S6 and 18S rRNA further support that ribosome synthesis and hence protein synthesis is impaired in adipocytes from diabetic subjects (Paper II).

FOXO1 itself is an important transcription factor that regulates important insulin signaling intermediaries. It is in fact interesting that FOXO1 can be regulated by TCF7L2 (260, 261), which is one of the strongest and consistently replicated GWAS results. Since we observed reduction in FOXO1 expression in adipocytes from diabetic individuals, it led us to hypothesize that downregulation of FOXO1 levels might be involved in reduction of insulin signaling proteins in the diabetic state. Consistent with our hypothesis, inhibition of FOXO1 with a dominant negative mutant reduced the protein levels of IR, GLUT4 and S6 in human adipocytes (Paper III). The most interesting finding in Paper III was that inhibition of FOXO1 induced network-wide insulin resistance in adipocytes that remarkably replicated the diabetic state. Looking for a mechanism that could explain this network-wide impairment of insulin signaling, we found that the abundance of mTOR and raptor and hence mTORC1 activity was reduced with FOXO1 inhibition. These results thus indicate that FOXO1 could be important to maintain mTORC1 activity in the adipocytes.

Additionally, FOXO1 also regulates expression of critical proteins such as transcriptional regulator PPAR γ and the adipokine adiponectin, both of which are crucial for adipocyte function. PPAR γ is not only integral for adipocyte differentiation but also lipid storage and metabolism. In fact, transgenic mice with adipocyte-specific activation of PPAR γ exhibited improved hepatic and peripheral insulin sensitivity and reduced hepatic steatosis under high fat diet without affecting adipogenesis, indicating the importance of adipocyte-specific

activity of PPAR γ to maintain whole body insulin sensitivity (253). Adiponectin is also involved in an intricate cross-talk with other insulin sensitive tissues to maintain systemic insulin sensitivity through its positive effects on hepatic and skeletal muscle lipid and glucose metabolism, vascular health, and its protective role in ectopic lipid accumulation (262). Thus, FOXO1 might also affect systemic insulin sensitivity through regulation of PPAR γ and adiponectin in human adipocytes.

It was also interesting that long-term inhibition of mTORC1 with rapamycin resulted in the reduction of FOXO1 protein levels comparable to the levels in adipocytes from diabetic individuals. This indicates that in addition to acute inhibition of FOXO1 activity via mTORC2, insulin can also maintain the protein levels of FOXO1 via mTORC1. FOXO1 and mTORC1 have been shown to crosstalk in a number of studies where FOXO1 has mostly been reported to inhibit mTORC1 activity. For example, FOXO1 activity has been reported to inhibit myogenesis in C2C12 myoblasts through inhibition of mTORC1 activity where activation of FOXO1 resulted in proteasomal degradation of mTOR components (263). FOXO1 has also been shown to upregulate Sestrin 3 expression in MEFs, which inhibits mTORC1 activity via AMPK activation of TSC2. The same study also reports upregulation of mTORC2 activity by FOXO1 through regulation of rictor expression (264). FOXO1 has also been shown to inhibit mTORC1 activity in *C. elegans* by inhibiting the expression of raptor (265). However, we have shown through the work in this thesis and previous publications that inhibition of either mTORC1 or FOXO1 in human adipocytes can induce system-wide insulin resistance similar to what is observed in adipocytes from diabetic individuals. It thus appears that FOXO1 and mTORC1 positively regulate each other in the human adipocytes to maintain their expression/activity and maintain the normal insulin signaling.

CONCLUSIONS AND FUTURE PERSPECTIVES

The findings in this thesis have expanded our understanding of the insulin signaling network extending into metabolic as well as transcriptional control by insulin in both normal and diabetic states in human adipocytes. We combined experimental data with mathematical modeling to define the molecular details of insulin regulation of ERK and FOXO1 and identified mechanisms that induce insulin resistance in adipocytes. We found that attenuation of mTORC1 activity can reproduce the diabetic phenotype in the adipocytes that extends into the ERK and FOXO1 pathways. At the same time, FOXO1 was also found to maintain mTORC1 activity. Our findings therefore point out a very interesting cross-talk between FOXO1 and mTORC1 to maintain insulin signaling in human adipocytes, and this interplay between FOXO1 and mTORC1 might play an important role in pathogenesis of insulin resistance in these cells. Our work also re-emphasizes that signaling pathways are not merely linear transmission of signals but are rather intricate web of cross-talks with negative and positive regulatory feedbacks.

Considering the importance of FOXO1 and mTORC1 to sustain normal insulin signaling in the adipocytes, it will be interesting to continue with in-depth investigation of how these two affect each other's activity. In this context, it is really interesting that dysregulated autophagy has been shown to increase lysosomal degradation of FOXO1 in peripheral T-cells (266). Increased autophagy due to attenuation of mTORC1 might play a role in the reduction in FOXO1 protein levels, in T2D or with long-term exposure to rapamycin in adipocytes. Or is this reduction mediated by ubiquitin-mediated degradation? Furthermore, cytoplasmic role of FOXO1 is an almost untouched area of investigation that might shed light on other interesting functions of FOXO1 in the cell.

There are many questions that remain unanswered. Is insulin resistance a protective mechanism to preserve cellular integrity due to lipid overload? If so, how does a fat cell sense its lipid accumulation limit before it becomes a threat to its own cellular integrity? Or is insulin resistance a consequence of dysregulation of a certain candidate gene/protein? Or are epigenetic changes involved in dysregulated gene/protein expression in adipocytes in the insulin resistant state. Enhancing insulin signaling in mature adipocytes might seem to be an interesting area for drug intervention. One can also consider other forms of intervention such as increasing lipid storage capacity through enhancement of adipogenesis or reducing the lipid load by increased fatty acid oxidation through browning of

white adipose tissue. However, the answers to the above mentioned questions determine the direction of drug intervention.

POPULAR SCIENCE ABSTRACT

The global epidemic of type 2 diabetes has been growing exponentially over the past two decades. It is one of the most prevalent metabolic disorders and is characterized by high glucose levels in blood. Insulin is the primary hormone that is responsible to maintain the blood glucose levels and is produced by the β -cells in response to food intake. Insulin stimulates the adipose tissue and the muscle to take up excess glucose and inhibits the release of glucose from the liver. Incapability of β -cells to produce sufficient insulin combined with the inability of different tissues to respond to normal levels of insulin, leads to development of T2D. This reduced insulin sensitivity which is also known as insulin resistance, develops in the insulin responsive tissues like muscle, adipose tissue and liver, much before the failure of β -cells. Despite extensive research in the field of diabetes, very little is known about the molecular mechanisms behind development of insulin resistance. Studies indicate that insulin resistance originates in the adipose tissue. It has been suggested that the inability of fat cells to store excess energy, is what diverts the lipid load to other tissues, leading to systemic insulin resistance.

Considering the important role of adipose tissue in maintaining energy homeostasis in the body, we investigated insulin signaling in fat cells isolated from fat biopsies from both normal and diabetic individuals. Our investigation was focused on the transcriptional control of insulin, which regulates the expression of different proteins in the fat cell. We analyzed several proteins in the insulin signaling network, their insulin sensitivity and activity, together with different perturbations in the network. The measured data from different proteins was integrated into a mathematical model for insulin signaling that could simulate data for both normal and diabetic individuals. With our work, we present for the first time, a comprehensive insulin signaling model for both metabolic and transcriptional control in human fat cells, both normally and in T2D. This model provides a unique understanding of insulin signaling and is a very helpful tool with diverse applications. The model provides the possibility to understand how insulin resistance develops in the fat cell. One can use the model to test how changes in one part of the pathway affects the entire network, for example in examining effects of drugs in different parts of the network. It can also be used to identify drug targets, by identifying parts of the pathway which can resolve insulin resistance.

With my work, we have also been able to narrow down the development of insulin resistance in fat cells to two players, a protein complex called mTORC1 and a transcription factor FOXO1. Inhibition of either of these two mimicked diabetic state in human fat cells. The similarity was displayed not only in insulin signaling but also at the protein levels that were found to be reduced in fat cells from diabetic individuals. This is a very exciting finding and understanding the mechanisms that cause dysregulation of these two players could provide clues to how insulin resistance develops in fat cells.

T2D is a treatable disease but not a curable one, which underlines the need to continue research efforts to understand the molecular details of how and why insulin resistance develops. There are the numerous questions that need to be addressed. Without a comprehensive picture, it is not possible to find a functional cure for the disease. We have taken a small step in this direction, by providing not only a network-wide understanding of insulin signaling in fat cells, but also by identifying possible mechanisms that impair insulin signaling in T2D. We believe that our findings provide potential areas for drug target intervention and possible areas for future investigations.

ACKNOWLEDGEMENT

The day I left my homeland is still very vivid in my mind. I set forth on a fabulous journey with many dreams, many expectations and many apprehensions. And undoubtedly, it has been a journey full of experiences and my time as a PhD student in Sweden has been one of the most rewarding ones. I have met wonderful people and made many new friends. I have had a memorable time during this period and learnt a lot, not only in research but also about life in general. There are quite a few people I would like extend my sincere and heartfelt gratitude to, without whom my time here wouldn't have been the same.

Peter Strålfors, it is one thing to be merely a supervisor and another to be a mentor. Thank you for your constant support and encouragement. You have always been protective about each one of us in the group. You have always believed in me and have inspired me to do better. Your mentorship has molded me into a better researcher. I have enjoyed your never ending enthusiasm about work. I have learnt a lot from your knowledge and perspective on different things.

Mats Söderström, thank you for sharing your technical knowledge and for your constant support. Be it science or TV series, you are an absolute storehouse of information.

Gunnar Cedersund, an absolute all-rounder, be it science or music. You have indeed given me new perspective on research with systems biology and also inspired me to manage my hobbies along with research.

Elin Nyman, you are the nicest person I have ever met. You bring an aura of exuberance that always lightens up the spirits of everyone around you. You have always been there to help me with my unending questions and problems, be it work-related or life-related. It is amazing how you stay calm in the most nerve racking situations. I really need to learn that from you. I will miss you terribly now that you will be leaving for Boston. I have loved working with you and look forward to continue to do that.

Åsa Jufvas, you are a friend I have earned for life. I remember that I used to be scared to talk to you when I started in the group. You invited me for Christmas dinner with your family and there began a friendship that I am very grateful for. I also want to give you the credit for being the real driving force that helped me to learn Swedish

so fast. I don't think that my time here on plan 12 would have been as much fun without you. I miss your company and your laughter echoing in our corridor.

Cecilia Jönsson, you are a wonderful person. I have loved talking to you whether it is about research or just to let out my stress or giving you suggestions on Indian middle names. I hope your love for Bollywood movies does not stop at dabangg and 3 idiots!

Cecilia Brännmark, I think you believe in me more than I do in myself. You have inspired me with your ambition and with the goodness in your heart. Thank you for believing in me and for all your efforts in trying to help me to get to a good place after my PhD. Hopefully that is Gothenburg ☺

Siri Fagerholm, you are one kind soul. You helped and guided me when I started in the group. You had been so patient with me and I must confess that you have not only been a good teacher but also a good friend. I have really enjoyed the times we spent together both in the lab and outside.

Yun Shen, my roommate and my "swimming partner". You are a very hard working and sincere person. I could always rely on you on any help I needed. How much we both miss our food was quite evident from our unending conversations about food.

Maria Turkina, the all-in-all of mass spec.....your expertise in proteomics has helped me a lot. I have enjoyed talking with you about anything and everything under the sky. No one can beat your sense of aesthetics.

Thank you to all the people on Floor 12. **Shadi Jafari**, I have bothered you with a lot of questions while I was writing my thesis. You have been a wonderful company. I have enjoyed our conversations about Swedish, about society, about similarity between Indian and Iranian food and culture and the list would go on... **Anita Öst**, your passion for science is very inspiring. It is so fun to talk to you. **Mattias Alenius**, you have been such a pleasant company. Your words of advice on writing the thesis and words of reassurance that I am doing fine have been very helpful and comforting.

Thorsell and Öst groups – Annika Thorsell, Lovisa Holm, Daniel Nätt, Riccardo Barchiesi, Asif Aziz, Esi Domi, Estelle Barbier, Lovisa Örkenby, Thomas, Unn Kugelberg, thank you for the lively discussions in the coffee room. Lunch and fika would have been so boring without you guys.

I also want to thank my present colleagues and collaborators especially, **Charlotta Olofsson, Mihaela Martis, Mika Gustafsson, Simon Söderholm, Rasmus Magnusson.**

During these years many people came and many have left but their memories will always stay in my heart: **Liza Alkhori, Sebastian Schultz, Anujaianthi Kuzhandaivel, Olivia Forsberg, Linnea Bergholm, David Julleson, Eva-Maria Ekstrand, Kataryne Rossitier**. I also want to thank my present and previous friends from floor 13: **Björn Ingelsson, Sara Tedesco, Christine Gallampois, Chamilly Evaldsson** for all the nice times together.

Thank you **Stefan Klintström, Charlotte Immerstrand, Anette Andersson** and all the **Forum Scientium members** for providing an amazing multidisciplinary platform, arranging social events, conferences and study visits all of which have been a learning experience for me. I would also like to thank **Preben Kjølhede**, the clinical staff and all patients who have provided us with fat biopsies for our research.

I would like to thank **Anna Weinhofer** and **Hakan Wiktander** for always being there to solve our problems.

I especially want to thank all my friends who have kept me strong and in good spirit. Thank you **Mayur, Sumit and Teja** who have always come forward to my help without any hesitation.

Sandeep, “my partner in crime”, you remind me of my brother and have been the one who takes my side, “most of the time”☺. **Binu, Neena, Varun, Dawn, Deepak, Shibi, Shani, Jithu and my darling Jenna**, I relish our times spent together. Our weekend fun, midnight birthday bashes, Binu and Jithu’s undending stories, your classic sense of humor Deepak, watching Peppa pig and my little pony with you Jenna, imaginary vacation planning on You Tube with you Shibi and of course Shani’s special mutton biryani. In fact, all of you have been my second family here.

Last but not the least, I wouldn’t have come this far without the love and support of my loving family.

Mamma and Daddy, thank you for always being there for me and always keeping me in your thoughts and prayers.

Mummy and Papa, thank you for believing in me and my dreams. You have always supported me in my decisions and taught me the virtues of life. I am what I am because of your love and sacrifice. **Monu**, you have always stood by me through my ups and downs. You are a self-made man and you inspire me with your hard work and determination.

Jacob, you are my sunshine and my strength. You have been with me in my laughter and my tears. Thank you for your unconditional love and support. You are my rock in this journey of life.

REFERENCES

1. Rutkowski JM, Stern JH, Scherer PE. The cell biology of fat expansion. *The Journal of cell biology*. 2015;208(5):501-12. Epub 2015/03/04.
2. Galic S, Oakhill JS, Steinberg GR. Adipose tissue as an endocrine organ. *Molecular and cellular endocrinology*. 2010;316(2):129-39. Epub 2009/09/03.
3. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*. 2001;414(6865):799-806. Epub 2001/12/14.
4. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature*. 2006;444(7121):847-53. Epub 2006/12/15.
5. International Diabetes Federation. IDF Diabetes Atlas, 7 ed. Brussels, Belgium. International Diabetes Federation. 2015.
6. World Health Organization <http://www.who.int/mediacentre/factsheets/fs310/en/>. Accessed July 2016.
7. Sun K, Kusminski CM, Scherer PE. Adipose tissue remodeling and obesity. *The Journal of clinical investigation*. 2011;121(6):2094-101. Epub 2011/06/03.
8. Wildman RP, Muntner P, Reynolds K, McGinn AP, Rajpathak S, Wylie-Rosett J, et al. The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering: prevalence and correlates of 2 phenotypes among the US population (NHANES 1999-2004). *Archives of internal medicine*. 2008;168(15):1617-24. Epub 2008/08/13.
9. Bluher M. The distinction of metabolically 'healthy' from 'unhealthy' obese individuals. *Current opinion in lipidology*. 2010;21(1):38-43. Epub 2009/11/17.
10. Karelis AD. To be obese—does it matter if you are metabolically healthy? *Nature reviews Endocrinology*. 2011;7(12):699-700. Epub 2011/10/20.
11. Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, et al. Dynamics of fat cell turnover in humans. *Nature*. 2008;453(7196):783-7. Epub 2008/05/06.
12. Franck N, Stenkula KG, Ost A, Lindstrom T, Stralfors P, Nystrom FH. Insulin-induced GLUT4 translocation to the plasma membrane is blunted in large compared with small primary fat cells isolated from the same individual. *Diabetologia*. 2007;50(8):1716-22.
13. Laurencikiene J, Skurk T, Kulyte A, Heden P, Astrom G, Sjolín E, et al. Regulation of Lipolysis in Small and Large Fat Cells of the Same Subject. *J Clin Endocr Metab*. 2011;96(12):E2045-E9.
14. Kloting N, Fasshauer M, Dietrich A, Kovacs P, Schon MR, Kern M, et al. Insulin-sensitive obesity. *Am J Physiol-Endoc M*. 2010;299(3):E506-E15.
15. Salans LB, Dougherty JW. The effect of insulin upon glucose metabolism by adipose cells of different size. Influence of cell lipid and protein content, age, and nutritional state. *The Journal of clinical investigation*. 1971;50(7):1399-410. Epub 1971/07/01.
16. Jernas M, Palming J, Sjöholm K, Jennische E, Svensson PA, Gabrielsson BG, et al. Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2006;20(9):1540-2. Epub 2006/06/07.
17. Lonn M, Mehlíg K, Bengtsson C, Lissner L. Adipocyte size predicts incidence of type 2 diabetes in women. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2010;24(1):326-31. Epub 2009/09/11.
18. Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE. Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. *Diabetologia*. 2000;43(12):1498-506. Epub 2001/01/11.
19. Andersson DP, Eriksson Hogling D, Thorell A, Toft E, Qvisth V, Naslund E, et al. Changes in subcutaneous fat cell volume and insulin sensitivity after weight loss. *Diabetes care*. 2014;37(7):1831-6. Epub 2014/04/25.
20. Attie AD, Scherer PE. Adipocyte metabolism and obesity. *Journal of lipid research*. 2009;50 Suppl:S395-9. Epub 2008/11/20.
21. Danforth E, Jr. Failure of adipocyte differentiation causes type II diabetes mellitus? *Nature genetics*. 2000;26(1):13. Epub 2000/09/06.

22. Hotamisligil GS. Inflammation and metabolic disorders. *Nature*. 2006;444(7121):860-7. Epub 2006/12/15.
23. Lumeng CN, Saltiel AR. Inflammatory links between obesity and metabolic disease. *The Journal of clinical investigation*. 2011;121(6):2111-7. Epub 2011/06/03.
24. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *The Journal of clinical investigation*. 2003;112(12):1821-30. Epub 2003/12/18.
25. Halberg N, Khan T, Trujillo ME, Wernstedt-Asterholm I, Attie AD, Sherwani S, et al. Hypoxia-inducible factor 1 α induces fibrosis and insulin resistance in white adipose tissue. *Molecular and cellular biology*. 2009;29(16):4467-83. Epub 2009/06/24.
26. Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, et al. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes*. 2007;56(4):901-11. Epub 2007/03/31.
27. Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *American journal of physiology Endocrinology and metabolism*. 2007;293(4):E1118-28. Epub 2007/08/02.
28. Chen B, Lam KS, Wang Y, Wu D, Lam MC, Shen J, et al. Hypoxia dysregulates the production of adiponectin and plasminogen activator inhibitor-1 independent of reactive oxygen species in adipocytes. *Biochemical and biophysical research communications*. 2006;341(2):549-56. Epub 2006/01/24.
29. Skurk T, Alberti-Huber C, Herder C, Hauner H. Relationship between adipocyte size and adipokine expression and secretion. *The Journal of clinical endocrinology and metabolism*. 2007;92(3):1023-33. Epub 2006/12/14.
30. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science*. 1993;259(5091):87-91. Epub 1993/01/01.
31. Amrani A, Jafarian-Tehrani M, Mormede P, Durant S, Pleau JM, Haour F, et al. Interleukin-1 effect on glycemia in the non-obese diabetic mouse at the pre-diabetic stage. *The Journal of endocrinology*. 1996;148(1):139-48. Epub 1996/01/01.
32. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100(12):7265-70. Epub 2003/05/21.
33. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *Journal of lipid research*. 2005;46(11):2347-55. Epub 2005/09/10.
34. Nishimura S, Manabe I, Nagasaki M, Seo K, Yamashita H, Hosoya Y, et al. In vivo imaging in mice reveals local cell dynamics and inflammation in obese adipose tissue. *The Journal of clinical investigation*. 2008;118(2):710-21. Epub 2008/01/19.
35. Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, 2nd, DeFuria J, Jick Z, et al. Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes*. 2007;56(12):2910-8. Epub 2007/09/13.
36. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *The Journal of clinical investigation*. 2007;117(1):175-84. Epub 2007/01/04.
37. Castoldi A, Naffah de Souza C, Camara NO, Moraes-Vieira PM. The Macrophage Switch in Obesity Development. *Frontiers in immunology*. 2015;6:637. Epub 2016/01/19.
38. Fujisaka S, Usui I, Bukhari A, Ikutani M, Oya T, Kanatani Y, et al. Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice. *Diabetes*. 2009;58(11):2574-82. Epub 2009/08/20.
39. Patsouris D, Li PP, Thapar D, Chapman J, Olefsky JM, Neels JG. Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals. *Cell metabolism*. 2008;8(4):301-9. Epub 2008/10/09.

40. Lee YH, Thacker RI, Hall BE, Kong R, Granneman JG. Exploring the activated adipogenic niche: interactions of macrophages and adipocyte progenitors. *Cell cycle*. 2014;13(2):184-90. Epub 2014/01/08.
41. Franssen R, Monajemi H, Stroes ES, Kastelein JJ. Obesity and dyslipidemia. *The Medical clinics of North America*. 2011;95(5):893-902. Epub 2011/08/23.
42. Kosteli A, Sugaru E, Haemmerle G, Martin JF, Lei J, Zechner R, et al. Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. *The Journal of clinical investigation*. 2010;120(10):3466-79. Epub 2010/09/30.
43. Boden G, She P, Mozzoli M, Cheung P, Gumireddy K, Reddy P, et al. Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-kappaB pathway in rat liver. *Diabetes*. 2005;54(12):3458-65. Epub 2005/11/25.
44. Itani SI, Ruderman NB, Schmieder F, Boden G. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. *Diabetes*. 2002;51(7):2005-11. Epub 2002/06/28.
45. Nguyen MT, Favellyukis S, Nguyen AK, Reichart D, Scott PA, Jenn A, et al. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. *The Journal of biological chemistry*. 2007;282(48):35279-92. Epub 2007/10/06.
46. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *The Journal of clinical investigation*. 1995;95(5):2409-15. Epub 1995/05/01.
47. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. *The Journal of clinical investigation*. 2003;112(12):1796-808. Epub 2003/12/18.
48. Wernstedt Asterholm I, Tao C, Morley TS, Wang QA, Delgado-Lopez F, Wang ZV, et al. Adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling. *Cell metabolism*. 2014;20(1):103-18. Epub 2014/06/17.
49. Kleemann R, van Erk M, Verschuren L, van den Hoek AM, Koek M, Wielinga PY, et al. Time-resolved and tissue-specific systems analysis of the pathogenesis of insulin resistance. *PloS one*. 2010;5(1):e8817. Epub 2010/01/26.
50. Lee YS, Li P, Huh JY, Hwang IJ, Lu M, Kim JJ, et al. Inflammation is necessary for long-term but not short-term high-fat diet-induced insulin resistance. *Diabetes*. 2011;60(10):2474-83. Epub 2011/09/14.
51. Stern JH, Scherer PE. Adipose tissue biology in 2014: Advances in our understanding of adipose tissue homeostasis. *Nature reviews Endocrinology*. 2015;11(2):71-2. Epub 2014/12/17.
52. Kim JJ, Huh JY, Sohn JH, Choe SS, Lee YS, Lim CY, et al. Lipid-overloaded enlarged adipocytes provoke insulin resistance independent of inflammation. *Molecular and cellular biology*. 2015;35(10):1686-99. Epub 2015/03/04.
53. Rosen ED, MacDougald OA. Adipocyte differentiation from the inside out. *Nature reviews Molecular cell biology*. 2006;7(12):885-96. Epub 2006/12/02.
54. Wu Z, Rosen ED, Brun R, Hauser S, Adelmant G, Troy AE, et al. Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Molecular cell*. 1999;3(2):151-8. Epub 1999/03/17.
55. Isakson P, Hammarstedt A, Gustafson B, Smith U. Impaired preadipocyte differentiation in human abdominal obesity: role of Wnt, tumor necrosis factor-alpha, and inflammation. *Diabetes*. 2009;58(7):1550-7. Epub 2009/04/09.
56. Gustafson B, Smith U. The WNT inhibitor Dickkopf 1 and bone morphogenetic protein 4 rescue adipogenesis in hypertrophic obesity in humans. *Diabetes*. 2012;61(5):1217-24. Epub 2012/03/27.
57. Hammarstedt A, Hedjazifar S, Jenndahl L, Gogg S, Grunberg J, Gustafson B, et al. WISP2 regulates preadipocyte commitment and PPARgamma activation by BMP4. *Proceedings of the National Academy of Sciences of the United States of America*. 2013;110(7):2563-8. Epub 2013/01/30.

58. Altinli S, Elevli M, Ozkul AA, Kara PG, Karsidag K, Dogru M. Insulin resistance and metabolic syndrome in children of parents with diabetes mellitus. *Journal of pediatric endocrinology & metabolism : JPEM*. 2007;20(3):431-6. Epub 2007/04/25.
59. Abate N, Chandalia M, Snell PG, Grundy SM. Adipose tissue metabolites and insulin resistance in nondiabetic Asian Indian men. *The Journal of clinical endocrinology and metabolism*. 2004;89(6):2750-5. Epub 2004/06/08.
60. Baier LJ, Hanson RL. Genetic studies of the etiology of type 2 diabetes in Pima Indians: hunting for pieces to a complicated puzzle. *Diabetes*. 2004;53(5):1181-6. Epub 2004/04/28.
61. Voight BF, Scott LJ, Steinthorsdottir V, Morris AP, Dina C, Welch RP, et al. Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. *Nature genetics*. 2010;42(7):579-89. Epub 2010/06/29.
62. Dupuis J, Langenberg C, Prokopenko I, Saxena R, Soranzo N, Jackson AU, et al. New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk. *Nature genetics*. 2010;42(2):105-16. Epub 2010/01/19.
63. Grant SF, Thorleifsson G, Reynisdottir I, Benediktsson R, Manolescu A, Sainz J, et al. Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. *Nature genetics*. 2006;38(3):320-3. Epub 2006/01/18.
64. Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D, et al. A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature*. 2007;445(7130):881-5. Epub 2007/02/13.
65. Gloyn AL, Braun M, Rorsman P. Type 2 diabetes susceptibility gene TCF7L2 and its role in beta-cell function. *Diabetes*. 2009;58(4):800-2. Epub 2009/04/02.
66. Norton L, Chen X, Fourcaudot M, Acharya NK, DeFronzo RA, Heikkinen S. The mechanisms of genome-wide target gene regulation by TCF7L2 in liver cells. *Nucleic Acids Res*. 2014;42(22):13646-61.
67. Smith U. TCF7L2 and type 2 diabetes--we WNT to know. *Diabetologia*. 2007;50(1):5-7. Epub 2006/11/15.
68. Christodoulides C, Lagathu C, Sethi JK, Vidal-Puig A. Adipogenesis and WNT signalling. *Trends Endocrin Met*. 2009;20(1):16-24.
69. Prasad RB, Groop L. Genetics of type 2 diabetes-pitfalls and possibilities. *Genes*. 2015;6(1):87-123. Epub 2015/03/17.
70. Manning AK, Hivert MF, Scott RA, Grimsby JL, Bouatia-Naji N, Chen H, et al. A genome-wide approach accounting for body mass index identifies genetic variants influencing fasting glycemic traits and insulin resistance. *Nature genetics*. 2012;44(6):659-69. Epub 2012/05/15.
71. Miki H, Yamauchi T, Suzuki R, Komeda K, Tsuchida A, Kubota N, et al. Essential role of insulin receptor substrate 1 (IRS-1) and IRS-2 in adipocyte differentiation. *Molecular and cellular biology*. 2001;21(7):2521-32. Epub 2001/03/22.
72. Pasquali L, Gaulton KJ, Rodriguez-Segui SA, Mularoni L, Miguel-Escalada I, Akerman I, et al. Pancreatic islet enhancer clusters enriched in type 2 diabetes risk-associated variants. *Nature genetics*. 2014;46(2):136-43. Epub 2014/01/15.
73. Ravelli GP, Stein ZA, Susser MW. Obesity in young men after famine exposure in utero and early infancy. *The New England journal of medicine*. 1976;295(7):349-53. Epub 1976/08/12.
74. Gale CR, Javaid MK, Robinson SM, Law CM, Godfrey KM, Cooper C. Maternal size in pregnancy and body composition in children. *The Journal of clinical endocrinology and metabolism*. 2007;92(10):3904-11. Epub 2007/08/09.
75. Shapiro AL, Kaar JL, Crume TL, Starling AP, Siega-Riz AM, Ringham BM, et al. Maternal diet quality in pregnancy and neonatal adiposity: the Healthy Start Study. *International journal of obesity*. 2016;40(7):1056-62. Epub 2016/05/03.
76. Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *The Journal of nutrition*. 2005;135(6):1382-6. Epub 2005/06/03.
77. Jufvas A, Sjodin S, Lundqvist K, Amin R, Vener AV, Stralfors P. Global differences in specific histone H3 methylation are associated with overweight and type 2 diabetes. *Clinical epigenetics*. 2013;5(1):15. Epub 2013/09/06.

78. Lee J, Saha PK, Yang QH, Lee S, Park JY, Suh Y, et al. Targeted inactivation of MLL3 histone H3-Lys-4 methyltransferase activity in the mouse reveals vital roles for MLL3 in adipogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(49):19229-34. Epub 2008/12/03.
79. Mikkelsen TS, Xu Z, Zhang X, Wang L, Gimble JM, Lander ES, et al. Comparative epigenomic analysis of murine and human adipogenesis. *Cell*. 2010;143(1):156-69. Epub 2010/10/05.
80. Roy S, Sala R, Cagliero E, Lorenzi M. Overexpression of fibronectin induced by diabetes or high glucose: phenomenon with a memory. *Proceedings of the National Academy of Sciences of the United States of America*. 1990;87(1):404-8. Epub 1990/01/01.
81. El-Osta A, Brasacchio D, Yao D, Pocai A, Jones PL, Roeder RG, et al. Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. *The Journal of experimental medicine*. 2008;205(10):2409-17. Epub 2008/09/24.
82. Brasacchio D, Okabe J, Tikellis C, Balcerzyk A, George P, Baker EK, et al. Hyperglycemia induces a dynamic cooperativity of histone methylase and demethylase enzymes associated with gene-activating epigenetic marks that coexist on the lysine tail. *Diabetes*. 2009;58(5):1229-36. Epub 2009/02/12.
83. Multhaup ML, Seldin MM, Jaffe AE, Lei X, Kirchner H, Mondal P, et al. Mouse-human experimental epigenetic analysis unmasks dietary targets and genetic liability for diabetic phenotypes. *Cell metabolism*. 2015;21(1):138-49. Epub 2015/01/08.
84. Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. *Nature reviews Molecular cell biology*. 2006;7(2):85-96. Epub 2006/02/24.
85. Brannmark C, Palmer R, Glad ST, Cedersund G, Stralfors P. Mass and information feedbacks through receptor endocytosis govern insulin signaling as revealed using a parameter-free modeling framework. *The Journal of biological chemistry*. 2010;285(26):20171-9. Epub 2010/04/28.
86. Brannmark C, Nyman E, Fagerholm S, Bergenholm L, Ekstrand EM, Cedersund G, et al. Insulin signaling in type 2 diabetes: experimental and modeling analyses reveal mechanisms of insulin resistance in human adipocytes. *The Journal of biological chemistry*. 2013;288(14):9867-80. Epub 2013/02/13.
87. Ost A, Svensson K, Ruishalme I, Brannmark C, Franck N, Krook H, et al. Attenuated mTOR signaling and enhanced autophagy in adipocytes from obese patients with type 2 diabetes. *Molecular medicine*. 2010;16(7-8):235-46. Epub 2010/04/14.
88. Gustavsson J, Parpal S, Karlsson M, Ramsing C, Thorn H, Borg M, et al. Localization of the insulin receptor in caveolae of adipocyte plasma membrane. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 1999;13(14):1961-71. Epub 1999/11/02.
89. Parpal S, Karlsson M, Thorn H, Stralfors P. Cholesterol depletion disrupts caveolae and insulin receptor signaling for metabolic control via insulin receptor substrate-1, but not for mitogen-activated protein kinase control. *The Journal of biological chemistry*. 2001;276(13):9670-8. Epub 2000/12/31.
90. Foti M, Porcheron G, Fournier M, Maeder C, Carpentier JL. The neck of caveolae is a distinct plasma membrane subdomain that concentrates insulin receptors in 3T3-L1 adipocytes. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104(4):1242-7. Epub 2007/01/18.
91. Marshall S, Green A, Olefsky JM. Evidence for recycling of insulin receptors in isolated rat adipocytes. *The Journal of biological chemistry*. 1981;256(22):11464-70. Epub 1981/11/25.
92. Fagerholm S, Ortegren U, Karlsson M, Ruishalme I, Stralfors P. Rapid insulin-dependent endocytosis of the insulin receptor by caveolae in primary adipocytes. *PLoS one*. 2009;4(6):e5985. Epub 2009/06/23.
93. Thirone AC, Huang C, Klip A. Tissue-specific roles of IRS proteins in insulin signaling and glucose transport. *Trends in endocrinology and metabolism: TEM*. 2006;17(2):72-8. Epub 2006/02/07.

94. Taniguchi CM, Ueki K, Kahn R. Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *The Journal of clinical investigation*. 2005;115(3):718-27. Epub 2005/02/16.
95. Bjornholm M, He AR, Attersand A, Lake S, Liu SC, Lienhard GE, et al. Absence of functional insulin receptor substrate-3 (IRS-3) gene in humans. *Diabetologia*. 2002;45(12):1697-702. Epub 2002/12/19.
96. Boucher J, Kleinridders A, Kahn CR. Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harbor perspectives in biology*. 2014;6(1). Epub 2014/01/05.
97. Gual P, Le Marchand-Brustel Y, Tanti JF. Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. *Biochimie*. 2005;87(1):99-109. Epub 2005/03/01.
98. Copps KD, White MF. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. *Diabetologia*. 2012;55(10):2565-82. Epub 2012/08/08.
99. Danielsson A, Ost A, Nystrom FH, Stralfors P. Attenuation of insulin-stimulated insulin receptor substrate-1 serine 307 phosphorylation in insulin resistance of type 2 diabetes. *The Journal of biological chemistry*. 2005;280(41):34389-92. Epub 2005/09/01.
100. Danielsson A, Nystrom FH, Stralfors P. Phosphorylation of IRS1 at serine 307 and serine 312 in response to insulin in human adipocytes. *Biochemical and biophysical research communications*. 2006;342(4):1183-7. Epub 2006/03/07.
101. Ost A, Danielsson A, Liden M, Eriksson U, Nystrom FH, Stralfors P. Retinol-binding protein-4 attenuates insulin-induced phosphorylation of IRS1 and ERK1/2 in primary human adipocytes. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2007;21(13):3696-704. Epub 2007/06/19.
102. Weigert C, Kron M, Kalbacher H, Pohl AK, Runge H, Haring HU, et al. Interplay and effects of temporal changes in the phosphorylation state of serine-302, -307, and -318 of insulin receptor substrate-1 on insulin action in skeletal muscle cells. *Molecular endocrinology*. 2008;22(12):2729-40. Epub 2008/10/18.
103. Werner ED, Lee J, Hansen L, Yuan M, Shoelson SE. Insulin resistance due to phosphorylation of insulin receptor substrate-1 at serine 302. *The Journal of biological chemistry*. 2004;279(34):35298-305. Epub 2004/06/17.
104. Harrington LS, Findlay GM, Gray A, Tolkacheva T, Wigfield S, Rebholz H, et al. The TSC1-2 tumor suppressor controls insulin-P13K signaling via regulation of IRS proteins. *The Journal of cell biology*. 2004;166(2):213-23. Epub 2004/07/14.
105. Pereira MJ, Palming J, Rizell M, Aureliano M, Carvalho E, Svensson MK, et al. mTOR inhibition with rapamycin causes impaired insulin signalling and glucose uptake in human subcutaneous and omental adipocytes. *Molecular and cellular endocrinology*. 2012;355(1):96-105. Epub 2012/02/16.
106. Giraud J, Leshan R, Lee YH, White MF. Nutrient-dependent and insulin-stimulated phosphorylation of insulin receptor substrate-1 on serine 302 correlates with increased insulin signaling. *The Journal of biological chemistry*. 2004;279(5):3447-54. Epub 2003/11/19.
107. Carlson CJ, White MF, Rondinone CM. Mammalian target of rapamycin regulates IRS-1 serine 307 phosphorylation. *Biochemical and biophysical research communications*. 2004;316(2):533-9. Epub 2004/03/17.
108. Rajan MR, Fagerholm S, Jonsson C, Kjolhede P, Turkina MV, Stralfors P. Phosphorylation of IRS1 at Serine 307 in Response to Insulin in Human Adipocytes Is Not Likely to be Catalyzed by p70 Ribosomal S6 Kinase. *PloS one*. 2013;8(4).
109. Copps KD, Hancer NJ, Opore-Ado L, Qiu W, Walsh C, White MF. Irs1 serine 307 promotes insulin sensitivity in mice. *Cell metabolism*. 2010;11(1):84-92. Epub 2010/01/16.
110. Copps KD, Hancer NJ, Qiu W, White MF. Serine 302 Phosphorylation of Mouse Insulin Receptor Substrate 1 (IRS1) Is Dispensable for Normal Insulin Signaling and Feedback Regulation by Hepatic S6 Kinase. *The Journal of biological chemistry*. 2016;291(16):8602-17. Epub 2016/02/06.

111. Dummer B, Tschopp O, Hynx D, Yang ZZ, Dirnhofer S, Hemmings BA. Life with a single isoform of Akt: mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. *Molecular and cellular biology*. 2006;26(21):8042-51. Epub 2006/08/23.
112. Gonzalez E, McGraw TE. The Akt kinases: isoform specificity in metabolism and cancer. *Cell cycle*. 2009;8(16):2502-8. Epub 2009/07/15.
113. Fayard E, Tintignac LA, Baudry A, Hemmings BA. Protein kinase B/Akt at a glance. *Journal of cell science*. 2005;118(Pt 24):5675-8. Epub 2005/12/13.
114. Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Current biology : CB*. 1997;7(4):261-9. Epub 1997/04/01.
115. Alessi DR. Discovery of PDK1, one of the missing links in insulin signal transduction. Colworth Medal Lecture. *Biochemical Society transactions*. 2001;29(Pt 2):1-14. Epub 2001/05/18.
116. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, et al. Mechanism of activation of protein kinase B by insulin and IGF-1. *The EMBO journal*. 1996;15(23):6541-51. Epub 1996/12/02.
117. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 2005;307(5712):1098-101. Epub 2005/02/19.
118. Scheid MP, Marignani PA, Woodgett JR. Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. *Molecular and cellular biology*. 2002;22(17):6247-60. Epub 2002/08/09.
119. Yang J, Cron P, Thompson V, Good VM, Hess D, Hemmings BA, et al. Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. *Molecular cell*. 2002;9(6):1227-40. Epub 2002/06/28.
120. Collins BJ, Deak M, Arthur JS, Armit LJ, Alessi DR. In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *The EMBO journal*. 2003;22(16):4202-11. Epub 2003/08/13.
121. Yang G, Murashige DS, Humphrey SJ, James DE. A Positive Feedback Loop between Akt and mTORC2 via SIN1 Phosphorylation. *Cell reports*. 2015;12(6):937-43. Epub 2015/08/04.
122. Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, et al. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell*. 2006;127(1):125-37. Epub 2006/09/12.
123. Liu P, Begley M, Michowski W, Inuzuka H, Ginzberg M, Gao D, et al. Cell-cycle-regulated activation of Akt kinase by phosphorylation at its carboxyl terminus. *Nature*. 2014;508(7497):541-5. Epub 2014/03/29.
124. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, et al. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Developmental cell*. 2006;11(6):859-71. Epub 2006/12/05.
125. Vadlakonda L, Dash A, Pasupuleti M, Anil Kumar K, Reddanna P. The Paradox of Akt-mTOR Interactions. *Frontiers in oncology*. 2013;3:165. Epub 2013/06/27.
126. Kumar A, Lawrence JC, Jr., Jung DY, Ko HJ, Keller SR, Kim JK, et al. Fat cell-specific ablation of rictor in mice impairs insulin-regulated fat cell and whole-body glucose and lipid metabolism. *Diabetes*. 2010;59(6):1397-406. Epub 2010/03/25.
127. Kondapaka SB, Zarnowski M, Yver DR, Sausville EA, Cushman SW. 7-hydroxystaurosporine (UCN-01) inhibition of Akt Thr308 but not Ser473 phosphorylation: a basis for decreased insulin-stimulated glucose transport. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2004;10(21):7192-8. Epub 2004/11/10.
128. Cai H, Dong LQ, Liu F. Recent Advances in Adipose mTOR Signaling and Function: Therapeutic Prospects. *Trends in pharmacological sciences*. 2016;37(4):303-17. Epub 2015/12/25.
129. Wang X, Proud CG. mTORC1 signaling: what we still don't know. *Journal of molecular cell biology*. 2011;3(4):206-20. Epub 2010/12/09.
130. Betz C, Hall MN. Where is mTOR and what is it doing there? *The Journal of cell biology*. 2013;203(4):563-74. Epub 2014/01/05.

131. Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nature cell biology*. 2007;9(3):316-23. Epub 2007/02/06.
132. Wang LF, Harris TE, Roth RA, Lawrence JC. PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. *Journal of Biological Chemistry*. 2007;282(27):20036-44.
133. Yoon SO, Roux PP. Rapamycin Resistance: mTORC1 Substrates Hold Some of the Answers. *Current Biology*. 2013;23(19):R880-R3.
134. Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. *Febs Lett*. 2010;584(7):1287-95.
135. Ng S, Wu YT, Chen B, Zhou J, Shen HM. Impaired autophagy due to constitutive mTOR activation sensitizes TSC2-null cells to cell death under stress. *Autophagy*. 2011;7(10):1173-86. Epub 2011/08/03.
136. Polak P, Cybulski N, Feige JN, Auwerx J, Ruegg MA, Hall MN. Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration. *Cell metabolism*. 2008;8(5):399-410. Epub 2008/12/03.
137. Bentzinger CF, Romanino K, Cloetta D, Lin S, Mascarenhas JB, Oliveri F, et al. Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy. *Cell metabolism*. 2008;8(5):411-24. Epub 2008/12/03.
138. Oh WJ, Jacinto E. mTOR complex 2 signaling and functions. *Cell cycle*. 2011;10(14):2305-16. Epub 2011/06/15.
139. Kumar A, Harris TE, Keller SR, Choi KM, Magnuson MA, Lawrence JC, Jr. Muscle-specific deletion of rictor impairs insulin-stimulated glucose transport and enhances Basal glycogen synthase activity. *Molecular and cellular biology*. 2008;28(1):61-70. Epub 2007/10/31.
140. Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, et al. Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. *Cell metabolism*. 2012;15(5):725-38. Epub 2012/04/24.
141. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Molecular cell*. 2006;22(2):159-68. Epub 2006/04/11.
142. Roskoski R, Jr. ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacological research*. 2012;66(2):105-43. Epub 2012/05/10.
143. Pelicci G, Lanfrancone L, Grignani F, McGlade J, Cavallo F, Forni G, et al. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell*. 1992;70(1):93-104. Epub 1992/07/10.
144. Volmat V, Camps M, Arkinstall S, Pouyssegur J, Lenormand P. The nucleus, a site for signal termination by sequestration and inactivation of p42/p44 MAP kinases. *Journal of cell science*. 2001;114(Pt 19):3433-43. Epub 2001/10/30.
145. Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouyssegur J. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *The EMBO journal*. 1999;18(3):664-74. Epub 1999/02/02.
146. Suga J, Yoshimasa Y, Yamada K, Yamamoto Y, Inoue G, Okamoto M, et al. Differential activation of mitogen-activated protein kinase by insulin and epidermal growth factor in 3T3-L1 adipocytes: a possible involvement of PI3-kinase in the activation of the MAP kinase by insulin. *Diabetes*. 1997;46(5):735-41. Epub 1997/05/01.
147. Liu H, Kublaoui B, Pilch PF, Lee J. Insulin activation of mitogen-activated protein (MAP) kinase and Akt is phosphatidylinositol 3-kinase-dependent in rat adipocytes. *Biochemical and biophysical research communications*. 2000;274(3):845-51. Epub 2000/08/05.
148. Stenkula KG, Said L, Karlsson M, Thorn H, Kjolhede P, Gustavsson J, et al. Expression of a mutant IRS inhibits metabolic and mitogenic signalling of insulin in human adipocytes. *Molecular and cellular endocrinology*. 2004;221(1-2):1-8. Epub 2004/06/30.

149. Yamauchi K, Pessin JE. Insulin receptor substrate-1 (IRS1) and Shc compete for a limited pool of Grb2 in mediating insulin downstream signaling. *The Journal of biological chemistry*. 1994;269(49):31107-14. Epub 1994/12/09.
150. Skolnik EY, Batzer A, Li N, Lee CH, Lowenstein E, Mohammadi M, et al. The function of GRB2 in linking the insulin receptor to Ras signaling pathways. *Science*. 1993;260(5116):1953-5. Epub 1993/06/25.
151. Murphy LO, Blenis J. MAPK signal specificity: the right place at the right time. *Trends in biochemical sciences*. 2006;31(5):268-75. Epub 2006/04/11.
152. Kayali AG, Austin DA, Webster NJ. Stimulation of MAPK cascades by insulin and osmotic shock: lack of an involvement of p38 mitogen-activated protein kinase in glucose transport in 3T3-L1 adipocytes. *Diabetes*. 2000;49(11):1783-93. Epub 2000/11/15.
153. Agouni A, Owen C, Czopek A, Mody N, Delibegovic M. In vivo differential effects of fasting, re-feeding, insulin and insulin stimulation time course on insulin signaling pathway components in peripheral tissues. *Biochemical and biophysical research communications*. 2010;401(1):104-11. Epub 2010/09/14.
154. Nijhout HF, Callier V. A new mathematical approach for qualitative modeling of the insulin-TOR-MAPK network. *Frontiers in physiology*. 2013;4:245. Epub 2013/09/26.
155. Nyman E, Cedersund G, Stralfors P. Insulin signaling - mathematical modeling comes of age. *Trends in endocrinology and metabolism: TEM*. 2012;23(3):107-15. Epub 2012/01/31.
156. Cedersund G, Roll J. Systems biology: model based evaluation and comparison of potential explanations for given biological data. *The FEBS journal*. 2009;276(4):903-22. Epub 2009/02/14.
157. Lu Z, Xu S, Joazeiro C, Cobb MH, Hunter T. The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. *Molecular cell*. 2002;9(5):945-56. Epub 2002/06/07.
158. Zehorai E, Yao Z, Plotnikov A, Seger R. The subcellular localization of MEK and ERK--a novel nuclear translocation signal (NTS) paves a way to the nucleus. *Molecular and cellular endocrinology*. 2010;314(2):213-20. Epub 2009/05/02.
159. Roy F, Laberge G, Douziech M, Ferland-McCollough D, Therrien M. KSR is a scaffold required for activation of the ERK/MAPK module. *Genes & development*. 2002;16(4):427-38. Epub 2002/02/19.
160. Ritt DA, Daar IO, Morrison DK. KSR regulation of the Raf-MEK-ERK cascade. *Methods in enzymology*. 2006;407:224-37. Epub 2006/06/08.
161. Roy M, Li Z, Sacks DB. IQGAP1 is a scaffold for mitogen-activated protein kinase signaling. *Molecular and cellular biology*. 2005;25(18):7940-52. Epub 2005/09/02.
162. Jufvas A, Rajan MR, Jonsson C, Stralfors P, Turkina M. Scaffolding Protein IQGAP1 - an Insulin-dependent Link between Caveolae and the Cytoskeleton in Primary Human Adipocytes? *The Biochemical journal*. 2016. Epub 2016/07/28.
163. DeFea KA, Zalevsky J, Thoma MS, Dery O, Mullins RD, Bunnett NW. beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *The Journal of cell biology*. 2000;148(6):1267-81. Epub 2000/03/22.
164. Ramos JW. The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. *The international journal of biochemistry & cell biology*. 2008;40(12):2707-19. Epub 2008/06/20.
165. Ferrell JE, Jr., Bhatt RR. Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase. *The Journal of biological chemistry*. 1997;272(30):19008-16. Epub 1997/07/25.
166. Toni T, Ozaki Y, Kirk P, Kuroda S, Stumpf MP. Elucidating the in vivo phosphorylation dynamics of the ERK MAP kinase using quantitative proteomics data and Bayesian model selection. *Molecular bioSystems*. 2012;8(7):1921-9. Epub 2012/05/05.
167. Wortzel I, Seger R. The ERK Cascade: Distinct Functions within Various Subcellular Organelles. *Genes & cancer*. 2011;2(3):195-209. Epub 2011/07/23.

168. Mendoza MC, Er EE, Blenis J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends in biochemical sciences*. 2011;36(6):320-8. Epub 2011/05/03.
169. Hoeflich KP, O'Brien C, Boyd Z, Cavet G, Guerrero S, Jung K, et al. In vivo antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2009;15(14):4649-64. Epub 2009/07/02.
170. Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science*. 1999;286(5445):1741-4. Epub 1999/11/27.
171. Winter JN, Jefferson LS, Kimball SR. ERK and Akt signaling pathways function through parallel mechanisms to promote mTORC1 signaling. *American journal of physiology Cell physiology*. 2011;300(5):C1172-80. Epub 2011/02/04.
172. Carriere A, Romeo Y, Acosta-Jaquez HA, Moreau J, Bonneil E, Thibault P, et al. ERK1/2 phosphorylate Raptor to promote Ras-dependent activation of mTOR complex 1 (mTORC1). *The Journal of biological chemistry*. 2011;286(1):567-77. Epub 2010/11/13.
173. Wang L, Gout I, Proud CG. Cross-talk between the ERK and p70 S6 kinase (S6K) signaling pathways. MEK-dependent activation of S6K2 in cardiomyocytes. *The Journal of biological chemistry*. 2001;276(35):32670-7. Epub 2001/06/30.
174. Roux PP, Shahbazian D, Vu H, Holz MK, Cohen MS, Taunton J, et al. RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *The Journal of biological chemistry*. 2007;282(19):14056-64. Epub 2007/03/16.
175. Asada S, Daitoku H, Matsuzaki H, Saito T, Sudo T, Mukai H, et al. Mitogen-activated protein kinases, Erk and p38, phosphorylate and regulate Foxo1. *Cellular signalling*. 2007;19(3):519-27. Epub 2006/11/23.
176. Leng Y, Steiler TL, Zierath JR. Effects of insulin, contraction, and phorbol esters on mitogen-activated protein kinase signaling in skeletal muscle from lean and ob/ob mice. *Diabetes*. 2004;53(6):1436-44. Epub 2004/05/27.
177. Osman AA, Hancock J, Hunt DG, Ivy JL, Mandarino LJ. Exercise training increases ERK2 activity in skeletal muscle of obese Zucker rats. *Journal of applied physiology*. 2001;90(2):454-60. Epub 2001/02/13.
178. Bi L, Chiang JY, Ding WX, Dunn W, Roberts B, Li T. Saturated fatty acids activate ERK signaling to downregulate hepatic sortilin 1 in obese and diabetic mice. *Journal of lipid research*. 2013;54(10):2754-62. Epub 2013/08/02.
179. Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, et al. Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. *The Journal of clinical investigation*. 2000;105(3):311-20. Epub 2000/02/17.
180. Krook A, Bjornholm M, Galuska D, Jiang XJ, Fahlman R, Myers MG, Jr., et al. Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. *Diabetes*. 2000;49(2):284-92. Epub 2000/06/27.
181. Carlson CJ, Koterski S, Sciotti RJ, Poccard GB, Rondinone CM. Enhanced basal activation of mitogen-activated protein kinases in adipocytes from type 2 diabetes: potential role of p38 in the downregulation of GLUT4 expression. *Diabetes*. 2003;52(3):634-41. Epub 2003/02/28.
182. Bandyopadhyay GK, Yu JG, Ofrecio J, Olefsky JM. Increased p85/55/50 expression and decreased phosphatidylinositol 3-kinase activity in insulin-resistant human skeletal muscle. *Diabetes*. 2005;54(8):2351-9. Epub 2005/07/28.
183. Gogg S, Smith U, Jansson PA. Increased MAPK activation and impaired insulin signaling in subcutaneous microvascular endothelial cells in type 2 diabetes: the role of endothelin-1. *Diabetes*. 2009;58(10):2238-45. Epub 2009/07/08.
184. Ozaki KI, Awazu M, Tamiya M, Iwasaki Y, Harada A, Kugisaki S, et al. Targeting the ERK signaling pathway as a potential treatment for insulin resistance and type 2 diabetes. *American journal of physiology Endocrinology and metabolism*. 2016;ajpendo 00445 2015. Epub 2016/02/11.
185. Hwang SL, Jeong YT, Li X, Kim YD, Lu Y, Chang YC, et al. Inhibitory cross-talk between the AMPK and ERK pathways mediates endoplasmic reticulum stress-induced insulin resistance in skeletal muscle. *British journal of pharmacology*. 2013;169(1):69-81. Epub 2013/02/05.

186. Banks AS, McAllister FE, Camporez JP, Zushin PJ, Jurczak MJ, Laznik-Bogoslavski D, et al. An ERK/Cdk5 axis controls the diabetogenic actions of PPARgamma. *Nature*. 2015;517(7534):391-5. Epub 2014/11/20.
187. Danielsson A, Fagerholm S, Ost A, Franck N, Kjolhede P, Nystrom FH, et al. Short-term overeating induces insulin resistance in fat cells in lean human subjects. *Molecular medicine*. 2009;15(7-8):228-34. Epub 2009/07/14.
188. Tang QQ, Otto TC, Lane MD. Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100(1):44-9. Epub 2002/12/28.
189. Eijkelenboom A, Burgering BM. FOXOs: signalling integrators for homeostasis maintenance. *Nature reviews Molecular cell biology*. 2013;14(2):83-97. Epub 2013/01/18.
190. Zhao Y, Wang Y, Zhu WG. Applications of post-translational modifications of FoxO family proteins in biological functions. *Journal of molecular cell biology*. 2011;3(5):276-82. Epub 2011/06/15.
191. Obsil T, Obsilova V. Structure/function relationships underlying regulation of FOXO transcription factors. *Oncogene*. 2008;27(16):2263-75. Epub 2008/04/09.
192. Nakae J, Park BC, Accili D. Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. *The Journal of biological chemistry*. 1999;274(23):15982-5. Epub 1999/05/29.
193. Shiota C, Woo JT, Lindner J, Shelton KD, Magnuson MA. Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. *Developmental cell*. 2006;11(4):583-9. Epub 2006/09/12.
194. Daitoku H, Sakamaki J, Fukamizu A. Regulation of FoxO transcription factors by acetylation and protein-protein interactions. *Biochimica et biophysica acta*. 2011;1813(11):1954-60. Epub 2011/03/15.
195. Wang F, Nguyen M, Qin FX, Tong Q. SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction. *Aging cell*. 2007;6(4):505-14. Epub 2007/05/25.
196. Matsuzaki H, Daitoku H, Hatta M, Aoyama H, Yoshimochi K, Fukamizu A. Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(32):11278-83. Epub 2005/08/04.
197. Jing E, Gesta S, Kahn CR. SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation. *Cell metabolism*. 2007;6(2):105-14. Epub 2007/08/08.
198. Matsuzaki H, Daitoku H, Hatta M, Tanaka K, Fukamizu A. Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100(20):11285-90. Epub 2003/09/19.
199. Aoki M, Jiang H, Vogt PK. Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101(37):13613-7. Epub 2004/09/03.
200. Kitamura YI, Kitamura T, Kruse JP, Raum JC, Stein R, Gu W, et al. FoxO1 protects against pancreatic beta cell failure through NeuroD and MafA induction. *Cell metabolism*. 2005;2(3):153-63. Epub 2005/09/13.
201. Nakae J, Biggs WH, 3rd, Kitamura T, Cavenee WK, Wright CV, Arden KC, et al. Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nature genetics*. 2002;32(2):245-53. Epub 2002/09/10.
202. Nakae J, Kitamura T, Kitamura Y, Biggs WH, 3rd, Arden KC, Accili D. The forkhead transcription factor Foxo1 regulates adipocyte differentiation. *Developmental cell*. 2003;4(1):119-29. Epub 2003/01/18.
203. Altomonte J, Richter A, Harbaran S, Suriawinata J, Nakae J, Thung SN, et al. Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice. *American journal of physiology Endocrinology and metabolism*. 2003;285(4):E718-28. Epub 2003/06/05.
204. Subauste AR, Burant CF. Role of FoxO1 in FFA-induced oxidative stress in adipocytes. *American journal of physiology Endocrinology and metabolism*. 2007;293(1):E159-64. Epub 2007/03/22.

205. Matsumoto M, Pocai A, Rossetti L, Depinho RA, Accili D. Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor Foxo1 in liver. *Cell metabolism*. 2007;6(3):208-16. Epub 2007/09/05.
206. I OS, Zhang W, Wasserman DH, Liew CW, Liu J, Paik J, et al. FoxO1 integrates direct and indirect effects of insulin on hepatic glucose production and glucose utilization. *Nature communications*. 2015;6:7079. Epub 2015/05/13.
207. Cheng Z, Guo S, Copps K, Dong X, Kolipara R, Rodgers JT, et al. Foxo1 integrates insulin signaling with mitochondrial function in the liver. *Nature medicine*. 2009;15(11):1307-11. Epub 2009/10/20.
208. Zhang K, Li L, Qi Y, Zhu X, Gan B, DePinho RA, et al. Hepatic suppression of Foxo1 and Foxo3 causes hypoglycemia and hyperlipidemia in mice. *Endocrinology*. 2012;153(2):631-46. Epub 2011/12/08.
209. Haeusler RA, Kaestner KH, Accili D. FoxOs function synergistically to promote glucose production. *The Journal of biological chemistry*. 2010;285(46):35245-8. Epub 2010/10/01.
210. Mooradian AD. Dyslipidemia in type 2 diabetes mellitus. *Nature clinical practice Endocrinology & metabolism*. 2009;5(3):150-9. Epub 2009/02/21.
211. Iozzo P, Turpeinen AK, Takala T, Oikonen V, Bergman J, Gronroos T, et al. Defective liver disposal of free fatty acids in patients with impaired glucose tolerance. *The Journal of clinical endocrinology and metabolism*. 2004;89(7):3496-502. Epub 2004/07/09.
212. Gross DN, van den Heuvel AP, Birnbaum MJ. The role of FoxO in the regulation of metabolism. *Oncogene*. 2008;27(16):2320-36. Epub 2008/04/09.
213. Haeusler RA, Han S, Accili D. Hepatic FoxO1 ablation exacerbates lipid abnormalities during hyperglycemia. *The Journal of biological chemistry*. 2010;285(35):26861-8. Epub 2010/06/25.
214. Haeusler RA, Hartil K, Vaitheesvaran B, Arrieta-Cruz I, Knight CM, Cook JR, et al. Integrated control of hepatic lipogenesis versus glucose production requires FoxO transcription factors. *Nature communications*. 2014;5:5190. Epub 2014/10/14.
215. Matsumoto M, Han S, Kitamura T, Accili D. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. *The Journal of clinical investigation*. 2006;116(9):2464-72. Epub 2006/08/15.
216. Glauser DA, Schlegel W. The emerging role of FOXO transcription factors in pancreatic beta cells. *The Journal of endocrinology*. 2007;193(2):195-207. Epub 2007/05/02.
217. Kitamura T. The role of FOXO1 in beta-cell failure and type 2 diabetes mellitus. *Nature reviews Endocrinology*. 2013;9(10):615-23. Epub 2013/08/21.
218. Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs WH, 3rd, Wright CV, et al. The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *The Journal of clinical investigation*. 2002;110(12):1839-47. Epub 2002/12/19.
219. Bouchi R, Foo KS, Hua H, Tsuchiya K, Ohmura Y, Sandoval PR, et al. FOXO1 inhibition yields functional insulin-producing cells in human gut organoid cultures. *Nature communications*. 2014;5:4242. Epub 2014/07/01.
220. Ai J, Duan J, Lv X, Chen M, Yang Q, Sun H, et al. Overexpression of FoxO1 causes proliferation of cultured pancreatic beta cells exposed to low nutrition. *Biochemistry*. 2010;49(1):218-25. Epub 2009/11/27.
221. Kamei Y, Miura S, Suzuki M, Kai Y, Mizukami J, Taniguchi T, et al. Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated Type I (slow twitch/red muscle) fiber genes, and impaired glycemic control. *The Journal of biological chemistry*. 2004;279(39):41114-23. Epub 2004/07/24.
222. Kitamura T, Kitamura YI, Funahashi Y, Shawber CJ, Castrillon DH, Kolipara R, et al. A Foxo/Notch pathway controls myogenic differentiation and fiber type specification. *The Journal of clinical investigation*. 2007;117(9):2477-85. Epub 2007/08/25.
223. Bastie CC, Nahle Z, McLoughlin T, Esser K, Zhang W, Unterman T, et al. FoxO1 stimulates fatty acid uptake and oxidation in muscle cells through CD36-dependent and -independent mechanisms. *The Journal of biological chemistry*. 2005;280(14):14222-9. Epub 2005/02/05.

224. Furuyama T, Kitayama K, Yamashita H, Mori N. Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscle during energy deprivation. *The Biochemical journal*. 2003;375(Pt 2):365-71. Epub 2003/06/25.
225. Kamei Y, Mizukami J, Miura S, Suzuki M, Takahashi N, Kawada T, et al. A forkhead transcription factor FKHR up-regulates lipoprotein lipase expression in skeletal muscle. *FEBS letters*. 2003;536(1-3):232-6. Epub 2003/02/15.
226. Nakae J, Cao Y, Oki M, Orba Y, Sawa H, Kiyonari H, et al. Forkhead transcription factor FoxO1 in adipose tissue regulates energy storage and expenditure. *Diabetes*. 2008;57(3):563-76. Epub 2007/12/29.
227. Kim JJ, Li P, Huntley J, Chang JP, Arden KC, Olefsky JM. FoxO1 haploinsufficiency protects against high-fat diet-induced insulin resistance with enhanced peroxisome proliferator-activated receptor gamma activation in adipose tissue. *Diabetes*. 2009;58(6):1275-82. Epub 2009/03/18.
228. Fan W, Imamura T, Sonoda N, Sears DD, Patsouris D, Kim JJ, et al. FOXO1 transrepresses peroxisome proliferator-activated receptor gamma transactivation, coordinating an insulin-induced feed-forward response in adipocytes. *The Journal of biological chemistry*. 2009;284(18):12188-97. Epub 2009/02/28.
229. Armoni M, Harel C, Karni S, Chen H, Bar-Yoseph F, Ver MR, et al. FOXO1 represses peroxisome proliferator-activated receptor-gamma1 and -gamma2 gene promoters in primary adipocytes. A novel paradigm to increase insulin sensitivity. *The Journal of biological chemistry*. 2006;281(29):19881-91. Epub 2006/05/04.
230. Zou P, Liu L, Zheng L, Liu L, Stoneman RE, Cho A, et al. Targeting FoxO1 with AS1842856 suppresses adipogenesis. *Cell cycle*. 2014;13(23):3759-67. Epub 2014/12/09.
231. Siersbaek R, Nielsen R, Mandrup S. PPARgamma in adipocyte differentiation and metabolism--novel insights from genome-wide studies. *FEBS letters*. 2010;584(15):3242-9. Epub 2010/06/15.
232. Gervois P, Torra IP, Fruchart JC, Staels B. Regulation of lipid and lipoprotein metabolism by PPAR activators. *Clinical chemistry and laboratory medicine*. 2000;38(1):3-11. Epub 2000/04/25.
233. Sugii S, Olson P, Sears DD, Saberi M, Atkins AR, Barish GD, et al. PPAR gamma activation in adipocytes is sufficient for systemic insulin sensitization. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(52):22504-9.
234. Hofmann C, Lorenz K, Colca JR. Glucose transport deficiency in diabetic animals is corrected by treatment with the oral antihyperglycemic agent pioglitazone. *Endocrinology*. 1991;129(4):1915-25. Epub 1991/10/01.
235. Wu Z, Xie Y, Morrison RF, Bucher NL, Farmer SR. PPARgamma induces the insulin-dependent glucose transporter GLUT4 in the absence of C/EBPalpha during the conversion of 3T3 fibroblasts into adipocytes. *The Journal of clinical investigation*. 1998;101(1):22-32. Epub 1998/02/14.
236. Chakrabarti P, Kandror KV. FoxO1 controls insulin-dependent adipose triglyceride lipase (ATGL) expression and lipolysis in adipocytes. *The Journal of biological chemistry*. 2009;284(20):13296-300. Epub 2009/03/20.
237. Kwon HS, Huang B, Unterman TG, Harris RA. Protein kinase B-alpha inhibits human pyruvate dehydrogenase kinase-4 gene induction by dexamethasone through inactivation of FOXO transcription factors. *Diabetes*. 2004;53(4):899-910. Epub 2004/03/30.
238. Cadoudal T, Distel E, Durant S, Fouque F, Blouin JM, Collinet M, et al. Pyruvate dehydrogenase kinase 4: regulation by thiazolidinediones and implication in glyceroneogenesis in adipose tissue. *Diabetes*. 2008;57(9):2272-9. Epub 2008/06/04.
239. Kim JY, Ahn SV, Yoon JH, Koh SB, Yoon J, Yoo BS, et al. Prospective study of serum adiponectin and incident metabolic syndrome: the ARIRANG study. *Diabetes care*. 2013;36(6):1547-53. Epub 2013/01/01.
240. Fisman EZ, Tenenbaum A. Adiponectin: a manifold therapeutic target for metabolic syndrome, diabetes, and coronary disease? *Cardiovascular diabetology*. 2014;13:103. Epub 2014/06/25.

241. Korner A, Kratzsch J, Gausche R, Schaab M, Erbs S, Kiess W. New predictors of the metabolic syndrome in children--role of adipocytokines. *Pediatric research*. 2007;61(6):640-5. Epub 2007/04/12.
242. Qiao L, Shao J. SIRT1 regulates adiponectin gene expression through Foxo1-C/enhancer-binding protein alpha transcriptional complex. *The Journal of biological chemistry*. 2006;281(52):39915-24. Epub 2006/11/09.
243. Morrison RF, Farmer SR. Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. *The Journal of biological chemistry*. 1999;274(24):17088-97. Epub 1999/06/08.
244. Fiorentino FP, Symonds CE, Macaluso M, Giordano A. Senescence and p130/Rb12: a new beginning to the end. *Cell research*. 2009;19(9):1044-51. Epub 2009/08/12.
245. Tremblay F, Gagnon A, Veilleux A, Sorisky A, Marette A. Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. *Endocrinology*. 2005;146(3):1328-37. Epub 2004/12/04.
246. Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, et al. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *The Journal of clinical investigation*. 2008;118(9):3065-74. Epub 2008/08/30.
247. Rastogi R, Jiang Z, Ahmad N, Rosati R, Liu Y, Beuret L, et al. Rapamycin induces mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1) expression through activation of protein kinase B and mitogen-activated protein kinase kinase pathways. *The Journal of biological chemistry*. 2013;288(47):33966-77. Epub 2013/10/16.
248. Chen XG, Liu F, Song XF, Wang ZH, Dong ZQ, Hu ZQ, et al. Rapamycin regulates Akt and ERK phosphorylation through mTORC1 and mTORC2 signaling pathways. *Molecular carcinogenesis*. 2010;49(6):603-10. Epub 2010/06/01.
249. Soares HP, Ni Y, Kisfalvi K, Sinnott-Smith J, Rozengurt E. Different patterns of Akt and ERK feedback activation in response to rapamycin, active-site mTOR inhibitors and metformin in pancreatic cancer cells. *PloS one*. 2013;8(2):e57289. Epub 2013/02/26.
250. Smith U. Impaired ('diabetic') insulin signaling and action occur in fat cells long before glucose intolerance--is insulin resistance initiated in the adipose tissue? *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity*. 2002;26(7):897-904. Epub 2002/06/25.
251. Morley TS, Xia JY, Scherer PE. Selective enhancement of insulin sensitivity in the mature adipocyte is sufficient for systemic metabolic improvements. *Nature communications*. 2015;6:7906. Epub 2015/08/06.
252. Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E, et al. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature*. 2001;409(6821):729-33. Epub 2001/02/24.
253. Sugii S, Olson P, Sears DD, Saberi M, Atkins AR, Barish GD, et al. PPARgamma activation in adipocytes is sufficient for systemic insulin sensitization. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(52):22504-9. Epub 2009/12/19.
254. Liu P, Gan W, Inuzuka H, Lazorchak AS, Gao D, Arojo O, et al. Sin1 phosphorylation impairs mTORC2 complex integrity and inhibits downstream Akt signalling to suppress tumorigenesis. *Nature cell biology*. 2013;15(11):1340-50. Epub 2013/10/29.
255. Julien LA, Carriere A, Moreau J, Roux PP. mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. *Molecular and cellular biology*. 2010;30(4):908-21. Epub 2009/12/10.
256. Maianu L, Keller SR, Garvey WT. Adipocytes exhibit abnormal subcellular distribution and translocation of vesicles containing glucose transporter 4 and insulin-regulated aminopeptidase in type 2 diabetes mellitus: implications regarding defects in vesicle trafficking. *The Journal of clinical endocrinology and metabolism*. 2001;86(11):5450-6. Epub 2001/11/10.
257. Kampmann U, Christensen B, Nielsen TS, Pedersen SB, Orskov L, Lund S, et al. GLUT4 and UBC9 protein expression is reduced in muscle from type 2 diabetic patients with severe insulin resistance. *PloS one*. 2011;6(11):e27854. Epub 2011/11/25.

258. Rondinone CM, Wang LM, Lonnroth P, Wesslau C, Pierce JH, Smith U. Insulin receptor substrate (IRS) 1 is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. *Proceedings of the National Academy of Sciences of the United States of America*. 1997;94(8):4171-5. Epub 1997/04/15.
259. Tonks KT, Ng Y, Miller S, Coster AC, Samocha-Bonet D, Iseli TJ, et al. Impaired Akt phosphorylation in insulin-resistant human muscle is accompanied by selective and heterogeneous downstream defects. *Diabetologia*. 2013;56(4):875-85. Epub 2013/01/25.
260. Oh KJ, Park J, Kim SS, Oh H, Choi CS, Koo SH. TCF7L2 Modulates Glucose Homeostasis by Regulating CREB- and FoxO1-Dependent Transcriptional Pathway in the Liver. *Plos Genet*. 2012;8(9).
261. Norton L, Chen X, Fourcaudot M, Acharya NK, DeFronzo RA, Heikkinen S. The mechanisms of genome-wide target gene regulation by TCF7L2 in liver cells. *Nucleic Acids Res*. 2014;42(22):13646-61. Epub 2014/11/22.
262. Stern JH, Rutkowski JM, Scherer PE. Adiponectin, Leptin, and Fatty Acids in the Maintenance of Metabolic Homeostasis through Adipose Tissue Crosstalk. *Cell metabolism*. 2016;23(5):770-84. Epub 2016/05/12.
263. Wu AL, Kim JH, Zhang C, Unterman TG, Chen J. Forkhead box protein O1 negatively regulates skeletal myocyte differentiation through degradation of mammalian target of rapamycin pathway components. *Endocrinology*. 2008;149(3):1407-14. Epub 2007/12/15.
264. Chen CC, Jeon SM, Bhaskar PT, Nogueira V, Sundararajan D, Tonic I, et al. FoxOs inhibit mTORC1 and activate Akt by inducing the expression of Sestrin3 and Rictor. *Developmental cell*. 2010;18(4):592-604. Epub 2010/04/24.
265. Jia K, Chen D, Riddle DL. The TOR pathway interacts with the insulin signaling pathway to regulate *C. elegans* larval development, metabolism and life span. *Development*. 2004;131(16):3897-906. Epub 2004/07/16.
266. Ishikura S, Iwaihara Y, Tanaka Y, Luo H, Nishi K, Doi K, et al. The Nuclear Zinc Finger Protein Zfat Maintains FoxO1 Protein Levels in Peripheral T Cells by Regulating the Activities of Autophagy and the Akt Signaling Pathway. *The Journal of biological chemistry*. 2016;291(29):15282-91. Epub 2016/05/27.

Papers

The articles associated with this thesis have been removed for copyright reasons. For more details about these see:

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-131421>