INSULIN SIGNALING DYNAMICS IN HUMAN ADIPOCYTES

MATHEMATICAL MODELING REVEALS MECHANISMS OF
INSULIN RESISTANCE IN TYPE 2 DIABETES

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

It always seems impossible until it's done

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ABSTRACT

Type 2 diabetes is characterized by raised blood glucose levels caused by an insufficient insulin control of glucose homeostasis. This lack of control is expressed both through insufficient release of insulin by the pancreatic beta-cells, and through insulin resistance in the insulin-responding tissues. We find insulin resistance of the adipose tissue particularly interesting since it appears to influence other insulin-responding tissues, such as muscle and liver, to also become insulin resistant.

The insulin signaling network is highly complex with cross-interacting intermediaries, positive and negative feedbacks, etc. To facilitate the mechanistic understanding of this network, we obtain dynamic, information-rich data and use model-based analysis as a tool to formally test different hypotheses that arise from the experimental observations. With dynamic mathematical models, we are able to combine knowledge and experimental data into mechanistic hypotheses, and draw conclusions such as rejection of hypotheses and prediction of outcomes of new experiments.

We aim for an increased understanding of adipocyte insulin signaling and the underlying mechanisms of the insulin resistance that we observe in adipocytes from subjects diagnosed with type 2 diabetes. We also aim for a complete picture of the insulin signaling network in primary human adipocytes from normal and diabetic subjects with a link to relevant clinical parameters: plasma glucose and insulin. Such a complete picture of insulin signaling has not been presented before. Not for adipocytes and not for other types of cells.

In this thesis, I present the development of the first comprehensive insulin signaling model that can simulate both normal and diabetic data from adipocytes – and that is linked to a whole-body glucose-insulin model. In the linking process we conclude that at least two glucose uptake parameters differ between the *in vivo* and *in vitro* conditions (Paper I). We also perform a model analysis of the early insulin signaling dynamics in rat adipocytes and conclude that internalization is important for an apparent reversed order of phosphorylation seen in these cells (Paper II). In the development of the first version of the comprehensive insulin signaling model, we introduce a key parameter for the diabetic state – an attenuated feedback (Paper III). We finally continue to build on the comprehensive model and include signaling to nuclear transcription via ERK and report substantial crosstalk in the insulin signaling network (Paper IV).

POPULÄRVETENSKAPLIG SAMMANFATTNING

Typ 2-diabetes är en vanligt förekommande sjukdom där kroppens vävnader förlorar sin känslighet mot hormonet insulin. Insulinets uppgift är att hålla en jämn blodsockernivå dygnet runt. Insulin utsöndras i samband med måltider och påverkar muskel- och fettvävnader att ta upp socker och levern att sluta producera socker. Det är viktigt att hålla en jämn blodsockernivå eftersom låga nivåer kan vara direkt dödligt och höga nivåer är skadligt för blodkärlen, vilket i sin tur kan leda till skador på flera av kroppens organ. Diabetiker har problem med blodsockernivån på grund av deras avtagande känslighet för insulin, så kallad insulinresistens. Denna resistens uppstår i lever-, muskel- och fettvävnader, men idag saknas kunskap om vilka molekylära mekanismer som ligger bakom insulinresistensen. Sådan kunskap ger en ökad förståelse för sjukdomsförloppet och är en hjälp att hitta nya läkemedel till de som är drabbade av diabetes.

Vi studerar insulinresistens i fettceller eftersom fetma och typ 2-diabetes är starkt sammankopplade. Studier pekar även på att fettceller är de celler som tidigast under sjukdomsförloppet drabbas av insulinresistens. Fettceller från diabetiker skiljer sig från fettceller från friska när vi behandlar dem med insulin och mäter aktiviteten hos de proteiner som sänder insulinsignalen vidare genom cellen. Vi kan använda insamlade data från flera proteiner från både friska och diabetiker samtidigt och på så sätt hitta olika möjliga platser i cellen där insulinresistensen uppstår. Eftersom det är svårt att kombinera stora mängder data använder vi program som kan simulera data med hjälp av matematiska modeller och beräkningsalgoritmer. Med dessa hjälpmedel kan vi testa olika hypoteser och komma fram till nya experiment som skulle vara värdefulla för att komma närmare vårt mål – att förklara hur insulinresistens uppstår i fettceller.

Vi har alltså skapat matematiska modeller för utvalda proteiner som aktiveras av insulin. Genom att kombinera dessa modeller med data från friska och diabetiker har vi funnit en möjlig förklaring till hur insulinresistens uppstår i fettceller. Förklaringen bygger på en nedreglering av en i friska celler förstärkande återkoppling. Denna återkoppling påverkar de flesta proteiner eftersom den ligger direkt nedströms om insulinreceptorn – som insulin binder till på utsidan av cellen. Nedregleringen gör alltså att de flesta proteiner som normalt svarar på insulin får ett lägre svar och på så sätt sprids insulinresistensen i hela cellen. Tack vare användandet av matematiska modeller kombinerat med data av hög kvalitet från

mänskliga celler, kan vi presentera denna unika systemförståelse av insulinresistens i typ-2 diabetes.

För att våra resultat också ska få en klinisk betydelse har vi kopplat samman våra matematiska modeller för insulinsignalering inom fettceller med modeller som beskriver blodsockernivå och insulinutsöndring för alla kroppens organ. För att kunna göra detta har vi formulerat krav så att insulinsvaret på fettcellsnivå ska motsvara insulinsvaret på fettvävnadsnivå. Dessa krav kunde vi inte uppfylla direkt med en enkel sammankoppling av modellerna. Men med hänsyn till skillnader i exempelvis blodflöde och cellhantering (som kan verka stressande) kan modellerna simuleras tillsammans för att observera hur fettcellen interagerar med andra organ och vävnader. Sammankopplingen underlättar också studier av hur läkemedels verkan sprids från celler där de har sin effekt, till resten av kroppens organ.

Det är vanligt att studera insulinresistens genom att göra experiment på celler från genetiskt förändrade råttor eller möss, alternativt celler som är odlade och kemiskt påverkade för att likna fettceller. Vi använder istället fettceller som är isolerade från fettvävnad donerad från människor som är diagnostiserade med typ 2-diabetes och jämför med fettvävnad från människor utan diabetesdiagnos. Vi har på så sätt en unik möjlighet att studera hur den mänskliga sjukdomen typ 2-diabetes påverkar fettcellers insulinsignalering och insulinresistens. I ett av mina delarbeten har vi tittat på den initiala insulinsignalen i fettceller från råtta. Där ser vi att aktiveringen av proteiner skiljer sig mellan fettceller från människa och fettceller från råtta. Vi använder även i denna studie matematiska modeller för att testa olika idéer om hur aktiveringen i fettceller från råtta ser ut.

Läkemedelsföretagen står inför en stor utmaning att hitta läkemedel som kan bota eller åtminstone lindra sjukdomsförloppet vid typ 2-diabetes. För att kunna upptäcka nya funktionella läkemedel behövs en ökad förståelse för bakomliggande cellulära mekanismer, både i friskt tillstånd och hos diabetiker. En sådan förståelse tror jag bara kan uppnås med ett systematiskt arbete där data samlas in från mänskligt material, för att analyseras med hjälp av matematiska modeller. Jag tror att resultaten presenterade i denna avhandling är ett litet steg i rätt riktning.

LIST OF ORIGINAL PAPERS

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

- I **Elin Nyman**, Cecilia Brännmark, Robert Palmér, Jan Brugård, Fredrik H Nyström, Peter Strålfors, and Gunnar Cedersund
A hierarchical whole body modeling approach elucidates the link between in vitro insulin signaling and in vivo glucose homeostasis
Journal of Biological Chemistry, 2011 Jul 22, 286(29): 26028-41
- II **Elin Nyman**, Siri Fagerholm, David Julleson, Peter Strålfors, and Gunnar Cedersund
Mechanistic explanations for counter-intuitive phosphorylation dynamics of the insulin receptor and IRS1 in response to insulin in murine adipocytes
FEBS Journal, 2012 Mar, 279(6): 987-999
- III Cecilia Brännmark*, **Elin Nyman***, Siri Fagerholm, Linnéa Bergenholm, Eva-Maria Ekstrand, Gunnar Cedersund, and Peter Strålfors
Insulin signaling in type 2 diabetes – experimental and modeling analyses reveal mechanisms of insulin resistance in human adipocytes
Journal of Biological Chemistry, 2013 Apr 5, 288(14): 9867-80
- IV **Elin Nyman***, Meenu Rajan*, Siri Fagerholm, Cecilia Brännmark, Gunnar Cedersund, and Peter Strålfors
The insulin-signaling network in human adipocytes, normally and in diabetes – role of signaling through ERK1/2
Submitted

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ABBREVIATIONS

AS160	Akt substrate of 160 kDa
EGF	epidermal growth factor
Elk1	ets-like gene 1
ERK	extracellular regulated kinase
FOXO	forkhead class O transcription factors
GLUT1	glucose transporter 1
GLUT4	glucose transporter 4
IGF-1	insulin-like growth factor-1
IR	insulin receptor
IRS1	insulin receptor substrate-1
MEK	mitogen activated ERK kinases
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin, complex 1
mTORC2	mammalian target of rapamycin, complex 2
ODE	ordinary differential equation
p90-RSK	p90 ribosomal S6 kinase
PDK1	phosphoinositide-dependent kinase-1
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B
PPAR γ	peroxisome proliferator-activated receptor γ
S6	ribosomal protein S6
S6K	p70 ribosomal S6 kinase
sem	standard error of the mean
Ser	serine
T2D	type 2 diabetes
Thr	threonine
TNF	tumor necrosis factor
Tyr	tyrosine

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INTRODUCTION

Diabetes is increasing worldwide with epidemic proportions. In developed countries, the prevalence of diabetes is 8-10 % in adults, and out of these almost 90 % have type 2 diabetes [1]. Type 2 diabetes (T2D) is a metabolic disease characterized by dysfunctions in the insulin control of energy homeostasis; both through a non-sufficient release of insulin, and an insulin resistance in the insulin-responding tissues [2]. The insulin resistance is initially compensated for by elevated release of insulin. When the increased release of insulin no longer can compensate for the insulin resistance in the target tissues, T2D can be diagnosed. T2D and the long-term complications of the disease cause individual suffering and are costly for the society since there is a lack of good treatments available. A main reason for the lack of treatments is that the underlying intracellular mechanisms to the disease are not fully understood.

We focus on the intracellular mechanisms behind insulin resistance in human adipocytes – the main cell population in the adipose (i.e. fat) tissue [3]. The insulin resistance in the adipose tissue results from an expanding storage of fat and is of particular interest, since it appears to influence other insulin-responding tissues, such as muscle and liver, to also become insulin resistant [2, 4]. The adipose tissue is thus one of the possible origins of T2D. The main task of the adipose tissue is to provide a reversible energy reservoir and thus to buffer for changing fluxes of lipids [5]. The adipocytes in the adipose tissue store lipids as triglycerides in a large lipid droplet that fills more than 95 % of the cell volume, and the nucleus is squeezed into the remaining volume (**Figure 1**). Adipocytes also function as an endocrine organ and produce and secrete proteins (adipokines) as signals to communicate with other cells/organs [6]. We study the intracellular insulin response in adipocytes obtained from the subcutaneous adipose tissue of normal subjects and patients diagnosed with T2D, in order to unravel the mechanistic origin of insulin resistance.

As we understand more about signaling networks like the insulin signaling network, we progressively realize their inborn complexity. The further we reach in the research of intracellular signaling mechanisms, the more signaling intermediaries, crosstalk, and feedbacks are discovered [7]. To add on to this complexity, different kinds of ligands, as well as ligand abundance, location, and dynamics give rise to different intracellular responses [8]. To handle all this complexity and dissect the information in experimental

data, mathematical modeling is coming of age as an important tool for data analysis [9]. We use mathematical modeling as a tool to get maximal information out of our obtained experimental data and also to more intelligently design new experiments.

Figure 1: The morphology of an adipocyte

Adipocytes store triglycerides in a large lipid droplet that covers most of the cytosolic volume. The adipocyte nucleus is squeezed towards the plasma membrane.

It is important to increase knowledge of insulin resistance and T2D at the intracellular level, but perhaps even more important is to increase our knowledge of how these intracellular signals interplay with signals from other cells and organs. T2D is indeed manifested at the whole-body systemic level, and to relate our knowledge of insulin signaling in primary adipocytes with knowledge of the *in vivo* situation, we have developed a method to combine our intracellular models with whole-body models of glucose homeostasis. With such combined models, intracellular changes can be translated to whole-body responses, which is useful to simulate the effect of different treatments.

My overall aim with this thesis is to increase the knowledge of the dynamics of the intracellular signaling events in adipocytes in response to insulin by using mathematical models as a tool for data analysis. More specifically, I aim for a systems understanding of insulin signaling and insulin resistance, based on internally consistent data from human cells. In order to obtain such a systems understanding, we have integrated the modeling in the experimental work such that continuously modeling depends on experimental data, and

experimental data depends on modeling. I also aim for a proper link between intracellular human data and whole-body clinical data. In Paper I, we create such a link between mathematical models based on intracellular insulin signaling data on one hand, and mathematical models describing the dynamic flows of insulin and glucose on the whole-body level on the other hand. We discover the requirement for additional mechanisms to be able to link the *in vitro* and *in vivo* mathematical models and simulate *in vitro* and *in vivo* data simultaneously. In Paper II, we study the early dynamic insulin response in rat adipocytes and compare with the situation in human adipocytes. We learn that insulin receptor internalization is important for the dynamic behavior of phosphorylation and we find different explanations to an apparent reversed order of peaks in the phosphorylation dynamics. In Paper III, we study the whole network of insulin signaling for metabolic control – from insulin binding to glucose uptake and to control of protein synthesis – in adipocytes from normal and T2D subjects. With data from all the different signaling intermediaries we obtain a new systems understanding of insulin resistance. We build on these results in Paper IV, where we include the insulin control of the ERK signaling branch into our comprehensive mathematical model of insulin signaling and T2D. We use a minimal modeling approach to understand ERK phosphorylation dynamics. We also study crosstalk between insulin signaling in control of ERK and insulin signaling for metabolic control.

I have limited the scope of this thesis to mathematical models of insulin signaling in target cells, such as adipocytes and muscle cells. There are several efforts in modeling other aspects of T2D, for example with focus on beta-cell function and clinical disease progression. For a complete understanding of T2D, the different pieces to the puzzle must be combined, and such a combination benefit from the use of mathematical models. For the interested reader, modeling different aspects of T2D is reviewed in [10].

MATHEMATICAL MODELING OF INTRACELLULAR SIGNALING

Intracellular signaling systems are not linear pathways, but complex networks with positive and negative feedback loops and cross-interacting intermediaries [11]. The complexity of signaling networks aggravates the interpretation and understanding of experimental observations [12]. We therefore use mathematical modeling as a tool in data analysis, to be able to more formally test different hypotheses (**Figure 2**).

Figure 2: The modeling approach used in this thesis

Mechanistic hypotheses, i.e. mathematical models, are used together with experimental data to draw conclusions in the form of rejections and core predications. Model parameters are estimated and if model simulations do not agree with data for any parameter set, the model is rejected and we start over with new mechanistic hypotheses. If instead there is an acceptable fit between model simulation and data for at least one parameter set, we keep the model and seek for predictions with low uncertainty, i.e. core predictions, for the found acceptable parameters. Core predictions can be tested experimentally, and new data is added to the estimation process. If there are no useful predictions with low uncertainty to be found, the simulation result can instead be used to decide which experiment to perform to reduce the uncertainty of the model, and thus be able to obtain core predictions in the next modeling cycle.

The hypotheses are created from prior knowledge of the signaling network and available experimental observations, and while testing these hypotheses we are able to draw conclusions in the form of rejected models and predictions with low uncertainty, i.e. core predictions [13-15]. Rejected models are models that are not sufficient to explain all experimental observations. However, more complex models might be sufficient. We thus use the rejected models to build on when we create new hypotheses to test. On the other hand, we use conclusions in the form of core predictions as a director in the decision of which new experiments to perform to gain further information about the system. In the following sections, the basic concepts and notations of our mathematical modeling approach are explained in detail.

ORDINARY DIFFERENTIAL EQUATIONS AND OTHER MODELING FRAMEWORKS

Models based on ordinary differential equations (ODEs) describe dynamic behaviors and are ideal to simulate time resolved data. ODE models include *states* that correspond to amounts or concentrations of substances such as specific signal mediating proteins; *reaction rates* that correspond to the dynamic transition between the states; *parameters* that correspond to kinetic rate constants, initial conditions, or scaling factors; and *output variables* that correspond to the experimental observations.

As an example, the simple model of insulin receptor activation by insulin (**Figure 3**) can be described with the following system of ODEs:

$$\begin{aligned}
 d/dt (IR_m) &= -v_{1a} - v_{1b} + v_{1f} + v_{1e} \\
 d/dt (IR_{ins}) &= v_{1a} - v_{1b} \\
 d/dt (IR_{m_YP}) &= v_{1b} + v_{1c} - v_{1d} - v_{1f} \\
 d/dt (IR_{i_YP}) &= v_{1c} - v_{1d} \\
 d/dt (IR_i) &= v_{1d} - v_{1e} \\
 d/dt (X) &= v_{2b} - v_{2a} \\
 d/dt (X_p) &= v_{2a} - v_{2b}
 \end{aligned}$$

where IR_m , IR_{ins} , IR_{m_YP} , etc are the states and v_{1a} , v_{1b} , v_{1f} , etc are the reaction rates (**Figure 3**). These rates are described by equations that depend on the states and kinetic parameters (k_{1a} , k_{1b} , etc), and the input parameter (insulin):

$$\begin{aligned}
 v_{1a} &= IR_m \cdot k_{1a} \cdot \text{insulin} \\
 v_{1b} &= k_{1b} \cdot IR_m \\
 v_{1c} &= IR_{ins} \cdot k_{1c}
 \end{aligned}$$

$v1c = IRm_YP \cdot k1c$
 $v1d = IRi_YP \cdot k1d \cdot Xp$
 $v1e = IRi \cdot k1e$
 $v1f = IRm_YP \cdot k1f$
 $v2a = X \cdot k2a \cdot IRi_YP$
 $v2b = Xp \cdot k2b$

The output variable in this simple model is the total amount of phosphorylated receptor scaled with a parameter for normalization to data:

output = $k_{scale} \cdot (IRm_YP + IRi_YP)$

To simulate the model all the kinetic and scaling parameter values must be specified, as well as the initial conditions of all states. These values are typically unknown, and have to be estimated (see next section: Parameter estimation).

Figure 3: Outline of the simple insulin receptor activation model with a negative feedback

Arrows represent reactions between states; dashed arrows represent activation of a reaction. The model states are: **IRm** (inactive insulin receptor in plasma membrane), **IRins** (insulin bound **IRm**), **IRm_YP** (tyrosine phosphorylated **IRm**), **IRi_YP** (internalized and tyrosine phosphorylated **IR**), **IRi** (internalized inactive **IR**), **X** (inactive feedback protein), **X_P** (active feedback protein).

There are situations when the ODE modeling framework is not the appropriate choice. For example when it comes to high-throughput data without time resolution, there are other modeling frameworks that are better suited (reviewed in [7]). The simplest modeling frameworks are entirely data-driven and consist of methods from statistics (for example clustering and principal component analysis). Also, modeling frameworks based on

Bayesian inference use statistical methods to calculate the most probable network of interactions between the measured variables [16, 17]. There is another category of modeling frameworks that is based on logic relations, like Boolean or Fuzzy boolean frameworks (reviewed in [18]). The logic model structures are also simple compared to ODE models, and do not include kinetic parameters to determine. Finally, there are model formulations that are more complex than ODE models, such as partial differential equations, which contain spatial resolution, and stochastic models, which describe random processes.

PARAMETER ESTIMATION

The values of model parameters in ODE models (for example kinetic rate constants) are hard to determine experimentally for most biological systems. Regarding parameters for the insulin signaling system, there have been efforts to measure and calculate parameters for the binding between insulin and the insulin receptor (IR) [19]. However, such detailed knowledge is not available for human adipocytes. Also, parameters for downstream insulin signaling have not been measured. We instead estimate values of the parameters using the model structure, the experimental observations, and somewhat realistic limits of the parameter values. We use an optimization algorithm to test different parameter values within the limits, and evaluate the agreement between the simulated output of the model and the observations using the sum of squares of the residuals weighted by the standard error of the mean (sem). We use a function in the Systems biology toolbox for Matlab [20] – *simannealingSBAO* – that combines a simulated annealing [21] with a downhill simplex algorithm [22]. Simulated annealing is designed not to find the global optimum, but a good enough approximation of that optimum in a large parameter space. In the design of the combined algorithm, worse solutions are accepted in the beginning of the optimization process, but the probability of accepting such solutions decrease with time according to a manually defined scheme. We use a modified version of the *simannealingSBAO* function that initiates multiple simplexes that are situated far away from each other in the space of parameters. With the multiple simplexes we are able to find multiple areas in the space of parameters – areas which potentially include additional acceptable parameters [13, 23].

We save all acceptable sets of parameters, i.e. all sets of parameters that together with the model produce a simulation that is in good agreement with the data according to a statistical χ^2 test. If no such set of parameters is found, the model is rejected. If at least one such set of parameters is found, we usually find thousands of slightly different as well as substantially different sets of acceptable parameters. To be able to display these thousands

sets of parameters, we pick the extreme acceptable parameter sets, i.e. the parameter sets that contain a maximal or minimal value. Therefore, if a model contains 12 parameters, there are 12 maximal and 12 minimal values (24 sets of parameter values). Among the acceptable parameters, we also pick the best and worst solutions, i.e. the maximal and minimal value of the sum of squares of the residuals (2 sets of parameter values), since these can give additional information that is interesting to display.

Simulations with the found acceptable parameters can be used to draw conclusion, or to decide upon new experiments to perform to reduce uncertainty in model predictions. Such experiments are usually focused on reducing the largest uncertainties in the model by measuring states or combination of states with large uncertainty in model predictions.

CONCLUSIONS: REJECTIONS AND CORE PREDICTIONS

We use two different conclusions as directions in the model/data analysis process: rejections and core predictions (**Figure 2**). A rejection of a model means that the model is not good enough to describe the available data. A rejection of a model is a final statement, as opposed to a statement that a proposed model can describe data. In other words, a proposed model is acceptable only until new data have shown the contrary, but a rejected model will continue to be rejected also with new data. Model development thus continues as long as there are things of interest to understand and measure in the system. Core predictions – predictions with low uncertainty – are the other kind of conclusion we use in the process of analyzing data with mathematical models. In practice, core predictions are simulated predictions using a representation of all found parameter values that agree with the experimental observations (**Figure 4**). It is crucial to take the uncertainty of predictions into account since the system we study is underdetermined and unique estimations of the parameter values are not possible. A core prediction can be understood as a property that must be fulfilled if the evaluated model structure should serve as an explanation to data. We use core predictions in the design of new experiments.

Figure 4: Example of how to use core predictions

(A) A simple model structure with states X1-X3 and reaction rates v1-v3. (B) A representation of all found acceptable parameter sets are simulated together with the model structure (lines) and experimental data (dots $\pm$ sem) for the state X2. (C) A core prediction for the state X3 that was not measured shows that $>40\%$ of total X must be in the state X3 for this model structure to explain the data for X2. (D) A core prediction for the ratio of the rates v3 and v2 shows that v2 is always faster than v3 since the ratio is always less than 1. The core prediction in (C) can possibly be tested experimentally to be able to reject or strengthen the belief in the model structure. Different lines represent simulations of different acceptable parameter sets.

In Paper IV, we use a new method to search for specific core predictions [14, 24]. With this method, we perform new parameter estimations where we search for sets of parameters that extend the core prediction as far as possible, with the side condition that there is still a good enough agreement with experimental data (**Figure 5**). This approach is valuable since alone the optimization algorithm possibly will miss acceptable solutions for complex optimization problems. The missed solutions in the direction of the core prediction are

crucial to find before performing new experiments, since we use core predictions as conclusions. These solutions are found more easily with this method.

Figure 5: Methods to search for a specific core prediction

(A) A simple model structure with states $X1$ and $X2$ and reaction rates $v1$ and $v2$, where $X2$ is degraded and thus the total amount of X is decreasing over time. (B) Experimental data (dots $\pm$ sem) and model simulations (lines) of $X2$. (C) To be able to fit the simple model with the $X2$ data, the total amount of X at 90 minutes must be < 60 % of starting total X (grey area) for all different acceptable parameter simulations (lines). The different lines represent a search for increasing values of total remaining X at 90 minutes but still with acceptable agreement to original $X2$ data. (D) The simulations of total remaining X at 90 minutes compared with agreement to original $X2$ data (dots). Blue lines intersect at 41, which is the χ^2 value for rejection, and 60, which is the % remaining X at 90 minutes.

THE INSULIN SIGNALING NETWORK

The hormone insulin regulates levels of both glucose and fatty acids in the circulation [25]. Under conditions of elevated blood glucose levels, insulin causes liver cells (hepatocytes), muscle cells (myocytes), and adipocytes to take up and store glucose as glycogen. Insulin also inhibits the release of the hormone glucagon and thereby inhibits the release of glucose from the liver. Other systemic effects of insulin are stimulation of amino acid uptake, protein and lipid synthesis, and inhibition of lipolysis.

Figure 6: A simplified view of insulin signaling in adipocytes without feedbacks and crosstalk

In response to insulin several proteins are activated and intracellular processes are initiated. These processes are for instance nuclear transcription, glucose uptake, and protein translation.

The intracellular insulin signaling network (reviewed in [26, 27]) is controlled through the binding of insulin to the insulin receptor (IR). Downstream signal mediators, such as the insulin receptor substrate-1 (IRS1), are recruited to IR followed by activation through phosphorylation at tyrosine (Tyr) residues. IRS1 is also phosphorylated by protein kinases

at serine (Ser) and threonine (Thr) residues that serve as negative or positive modifiers of insulin signaling. The subsequent signaling cascades involve phosphoinositide 3-kinase (PI3K), phospho-inositide-dependent kinase-1 (PDK1), protein kinase B (PKB), and mammalian target of rapamycin (mTOR) (**Figure 6**). Insulin signaling results in several intracellular responses such as nuclear transcription, protein translation, and glucose uptake.

INSULIN BINDING TO THE INSULIN RECEPTOR

The insulin receptor (IR) is a transmembrane protein with two extracellular α -subunits that bind insulin molecules and two transmembrane β -subunits with cytosolic protein kinase activity [28]. Upon insulin binding, IR autophosphorylates on Tyr residues [29], which results in IR kinase activity towards other substrates, and a downstream signaling cascade is thereby initiated (**Figure 7**).

Figure 7: The insulin receptor

Insulin binding to the insulin receptor α -subunits result in autophosphorylation of the β -subunits.

The IR-insulin binding has been studied in competition and dissociation experiments to unravel the characteristics of IR and the mechanisms of binding and activation [19]. The data have been analyzed using mathematical modeling approaches and the important findings are: i) several insulin molecules can bind to each receptor [19, 30-33], ii) IR contains heterogeneous binding sites with different affinities for insulin [19, 29-31, 34-36], and iii) these different affinities can be explained by a specific interaction between the two α -subunits after insulin binding [19, 30, 31, 36]. Presently, the only mathematical model that can simulate all complex characteristics in insulin binding data is a comprehensive model by Kiselyov *et al.* [19] that is based on advanced biochemical structural information about IR. We have linked the Kiselyov insulin binding model [19] with our own developed

models of downstream insulin signaling (Paper I). Insulin-IR binding experiments are mostly performed in cell lines, but there are also some limited data from human adipocytes. The insulin receptor of human adipocytes have a high affinity for insulin [37]. Also, human adipocytes show no detectable IR degradation during the first 45 minutes of insulin stimulation [38].

INSULIN RECEPTOR INTERNALIZATION AND ACTIVATION OF IRS1

After insulin binding and activation the insulin receptor will be rapidly internalized into the cell via small invaginations of the plasma membrane (caveolae) [39] (**Figure 8**). The internalized IR dissociates from insulin and dephosphorylation occurs before the inactive form of IR is recycled back to the plasma membrane [40, 41]. The internalization and recycling of IR have been studied using mathematical modeling approaches in [13, 42-48]. The insulin receptor internalization and recycling are involved in creating an overshoot in the insulin response which is observed in IR autophosphorylation as well as in the downstream phosphorylation of IRS1 [13, 42]. This overshoot in the insulin response have been thoroughly studied in human adipocytes with a combined experimental and mathematical modeling approach [13, 42]. Different feedback mechanisms, such as insulin degradation and internalization, were proposed as explanations for the observed overshoot in the insulin response, but no single feedback mechanism could explain all observations. However, IR internalization was shown to be a necessary feedback mechanism. A mathematical model involving receptor internalization in combination with another feedback, which results in dephosphorylation of internalized IR, was proposed and that model could explain all observations [13]. We used this final proposed mathematical model for IR-IRS1 in the minimal model analyses in Paper I and II and further developed the model in Paper III.

In Paper II, we studied a similar overshoot in the insulin response at the level of IR and IRS1 in data from rat adipocytes. In rat adipocytes, IRS1 is not co-localized with IR in caveolae in the absence of insulin, as is the case in human adipocytes [49, 50]. We discovered that mathematical models describing data from rat adipocytes also need internalization of IR to explain data. However, the IR-IRS1 phosphorylation dynamics in rat adipocytes was different from the human data. In rat adipocytes, the peak of IRS1 phosphorylation apparently occurs earlier than the peak of IR phosphorylation, which is a counter-intuitive behavior since IRS1 is phosphorylated downstream of IR. It turned out that this counter-intuitive behavior can be explained simply by looking at the data in

another way. If the IRS1 response to insulin is decreasing before the IR response to insulin, it will look like the IRS1 response occurs earlier than the IR response even if the increase in response is simultaneous for IR and IRS1, because the response is normalized to the respective maximal values.

Figure 8: Insulin receptor internalization

After insulin binding and activation of the insulin receptor, small invaginations (caveolae) containing the insulin receptor will release from the plasma membrane.

In Paper II, we used a conclusive mathematical modeling approach and rejected explanations that were not sufficient to describe the reversed order of phosphorylation. With this approach we concluded that two pools of phosphorylated IR – a faster responding pool in the plasma membrane and a slower responding internalized pool – could explain this observation. The faster responding pool transmitted a fast signal to IRS1 while the slower pool was the dominating IR pool. However, this explanation required a large pool of internalized receptors, which we have previously shown is not the case [39]. A modified two pool hypothesis with limiting IRS1 concentration and specific IR-IRS1 binding could explain the reversed order of peaks of IR and IRS1. We also concluded that internalization together with a negative feedback downstream of IRS1 to the phosphorylation of IRS1 could explain the reversed order of peaks and also all our available phosphorylation and internalization data from rat adipocytes. This negative feedback model for rat adipocyte IR-IRS1 signaling in combination with the human adipocyte IR-IRS1 model in [13] provided an even better fit with rat adipocyte data (Paper II). The combined model can thus simulate IR-IRS1 signaling in both human and rat adipocytes, using only a change in parameter values.

PHOSPHORYLATION OF PKB BY PDK1 AND mTORC2

Phosphorylated IRS1 activates PI3K that increases the concentration of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) in the plasma membrane. PIP3 continues the signaling cascade by recruiting PKB and PDK1 to the plasma membrane [51] where PDK1 phosphorylates PKB at Thr 308 [52, 53]. Also, mTOR complex 2 (mTORC2), phosphorylates PKB at Ser 473 [54]. Both phosphorylations are needed in order for PKB to be fully active and specific to its substrates [55]. Targets of PKB include glycogen synthase kinase-3 (GSK3), which is involved in control of glycogen synthesis, and AS160, which is involved in the control of glucose uptake [56] (**Figure 9**).

Figure 9: Phosphorylation of PKB

Phosphorylation of PKB by PDK1 at Thr 308 and by mTORC2 at Ser 473 are both needed for PKB to receive full kinase activity. Important targets of active PKB are the glycogen synthase kinase GSK3, AS160 involved in glucose transporter translocation, mTORC1 which is a signaling hub involved in many cellular processes, and the transcription factor FOXO.

mTORC2 is a protein complex that consists of several proteins including mTOR and Rictor. Knockout mice lacking the Rictor protein (thus lacking mTORC2) in the adipose tissue have larger organs than normal mice and raised levels of insulin-like growth factor-1 (IGF-1) indicating a central role of mTORC2 in adipose tissue in control of systemic growth and metabolism [57]. Phosphorylation of mTOR at Ser 2481 have been shown to be

specific for mTORC2 [58]. But the dynamics of mTOR Ser 2481 phosphorylation is different from the dynamics of PKB phosphorylation at Ser 473 in the HeLa cell line [59]. The authors use a mathematical modeling approach and cannot simulate PKB Ser 473 phosphorylation data using inputs only from mTORC2, but need to include an extra unknown PKB Ser 473 kinase “PDK2”. However, the authors constrain their model to a strict ordering of phosphorylations at PKB, i.e. the Ser 473 phosphorylation is only allowed to occur after the Thr 308 phosphorylation. This strict order of phosphorylation is not supported by the literature, instead the two phosphorylations seem to be independent of each other [60]. Without this constrain there might be another solution were no longer the “PDK2” kinase is needed for the model to explain the PKB Ser 473 phosphorylation data. Another potential issue with the measurements of mTORC2 activity in [59] is that the specificity for mTORC2 phosphorylation of mTOR at Ser 2481 is questionable [61]. This phosphorylation may occur both in mTORC1 and mTORC2.

In the development of our insulin signaling model in Paper III, we implement phosphorylation of PKB at Thr 308 and Ser 473 independent of each other. The phosphorylation of PKB at Thr 308 via IRS1 and phosphorylation of PKB at Ser 473 via mTORC2 is enough to describe our data for normal subjects reasonably well.

REGULATORY FEEDBACKS CONTROL INSULIN SIGNALING VIA IRS1

The insulin signal is controlled with positive and negative regulations via several different protein kinases that phosphorylate Ser and Thr residues of IRS1. The protein complex mTORC1 is involved in this regulatory feedback signaling (reviewed in [62]). mTORC1 has a broad kinase activity and phosphorylates S6 kinase (S6K), involved in control of protein translation, as well as many other substrates with different affinities [63]. mTORC1 is specifically inhibited by the drug rapamycin. 3T3-L1 cells (a mouse derived fibroblast cell line differentiated to adipocyte-like cells) treated with rapamycin show decreased insulin stimulated IRS1 phosphorylation at several Ser phosphorylation residues including Ser 307 [64, 65]. The same decreased phosphorylation of IRS1 at Ser 307 in response to rapamycin treatment is also seen *in vivo* in rat muscle and adipose tissue (corresponding residue in rat and mice is Ser 302) [64]. Also in isolated human adipocytes, insulin-stimulated phosphorylation of IRS1 at Ser 307 is affected by rapamycin treatment [66, 67]. The phosphorylation of IRS1 at Ser 307 has been suggested to occur downstream of S6K, but we have shown that it is unlikely that S6K is the kinase for phosphorylation of IRS1 at Ser 307, at least in human adipocytes [68]. We have observed a positive effect of

phosphorylation of IRS1 at Ser 307 on the phosphorylation of IRS1 at Tyr in human adipocytes: rapamycin reduces also the phosphorylation of IRS1 at Tyr [66]. There are contradicting reports from experiments in cell lines, where phosphorylation of IRS1 at Ser 307 has a negative effect on insulin signaling [69]. However, experiments in cell lines have also shown that replacement of Ser 307 with alanine reduced the insulin-stimulated Tyr phosphorylation of IRS1 and reduced the insulin-stimulated phosphorylation of S6K [70], indicating a positive effect of the Ser 307-phosphorylation on IRS1 phosphorylation at Tyr residues and therefore on downstream signaling.

Figure 10: Feedbacks from mTORC1 to phosphorylation of IRS1 at Ser 307 and Ser 312

Phosphorylation at these residues have been shown to have positive and negative effects on the level of IRS1 phosphorylation at Tyr residues and hence the intracellular signaling

Ser 312 (Ser 307 in rat and mice) is another IRS1 residue phosphorylated downstream of mTORC1 [71] that has been well studied (**Figure 10**). Phosphorylation of this residue seemed to have a negative effect on IRS1 phosphorylation at Tyr by blocking interactions between IR and IRS1 and thus inhibiting insulin signaling [72]. However, knock-in mice with IRS1 Ser 312 replaced with alanine have impaired muscle insulin signaling [73] indicating a positive role in insulin signaling of phosphorylation of IRS1 at Ser 312 *in vivo* in mice.

In our comprehensive dynamic model of insulin signaling (Paper III-IV), we include phosphorylation of IRS1 at Ser 307 directly downstream of mTORC1 (**Figure 10**). We implement this phosphorylation to be independent of the phosphorylation of IRS1 at Tyr

and therefore we do not constrain the model parameters for Ser 307 phosphorylation to have a positive or negative effect on phosphorylation of IRS1 at Tyr. The best fit with data is obtained with a positive effect from IRS1 Ser 307 to Tyr phosphorylation (Paper III).

GLUCOSE TRANSPORTER TRANSLOCATION IN RESPONSE TO INSULIN TO ENHANCE GLUCOSE UPTAKE

The molecular regulation of glucose transport over the plasma membrane by insulin occurs in several steps: translocation of GLUT4 vesicles from intracellular storage compartments to the cell surface, tethering, i.e. the first interaction with the plasma membrane, docking of vesicles, fusion where the vesicles merge with the plasma membrane, and finally removal of GLUT4 from the cell surface (reviewed in [74]). The knowledge of these steps has evolved during the years and mathematical models of the translocation process have been used as tools to understand data for more than 20 years [75-82]. Quon [76] presented a simple two pool, two parameter model based on observations of glucose transporter translocation in rat adipocytes. Holman *et al.* [77] showed that at least three pools of GLUT4, including two intracellular pools, was needed to simulate all available GLUT4 translocation data from rat adipocytes and 3T3-L1 adipocytes. With four or more pools of GLUT4 the authors obtained a transient simulation of GLUT4 appearance at the cell surface [77]. Later, a combined experimental and modeling approach [80] discovered that not all intracellular GLUT4 is ready to be released to the plasma membrane and showed that there are different responses to insulin for the different pools [80]. Stenkula *et al.* [81] studied transfected rat adipocytes and found single GLUT4 molecules, but also functional clusters of GLUT4, in the plasma membrane. These clusters were introduced in the Holman model [77], and the combined dynamic model is in agreement with the presented dynamic data of GLUT4 vesicle fusion events and simulates the transient behavior of GLUT4 appearance [81].

AS160, a substrate of PKB, is involved in transmission of the insulin signal to translocation of GLUT4-containing vesicles in the cytosol to the plasma membrane [83, 84] (**Figure 11**). To unravel which steps of the translocation process that are regulated by AS160, Brewer *et al.* [82] silenced AS160 in 3T3-L1 adipocytes combined with inhibitors of PKB and found that a PKB substrate other than AS160 regulates the transfer from a non-cycling GLUT4 pool to a cycling pool and that AS160 regulates the reverse action. These results were tested and confirmed with mathematical modeling.

The translocation of GLUT4 differs between human and rat adipocytes. In human adipocytes, about 25 % of GLUT4 is located in the plasma membrane in the basal state and this amount increases approximately 2-fold with insulin stimulation [85]. In rat adipocytes, there is also around 50 % of GLUT4 at the plasma membrane after insulin stimulation, but the basal amount is less than 5 % of total GLUT4 [86]. Also in 3T3-L1 adipocytes the GLUT4 content at the plasma membrane increases more than 10-fold with insulin due to low basal content [87].

Figure 11: Mechanisms of glucose transporter translocation

Translocation of the insulin-regulated glucose transporter (GLUT4) from cytosolic vesicles to the plasma membrane upon insulin stimulation is mediated via PKB and AS160.

Our developed models of glucose uptake in Paper I and Paper III-IV contain no detailed mechanisms of glucose transporter translocation, since we do not have detailed information about these mechanisms from human adipocytes. Instead AS160 simply causes GLUT4 to translocate to the plasma membrane where glucose uptake is proportional to the amount of GLUT4 molecules present and to the glucose concentration used in the experiment, with saturation applied to the glucose concentration. We use the steady state observations of GLUT4 content in the plasma membrane [85] and our own measurements of glucose uptake for different concentrations of insulin and glucose (Paper I) to find values of the parameters. We also add a constant GLUT1 glucose uptake, since the insulin-insensitive GLUT1 glucose transporter also is present in adipocytes. GLUT1 is responsible for the basal glucose uptake required for the basal needs of the adipocytes and other cells. The contributions of GLUT1 and GLUT4 to glucose uptake can be examined with a drug that mostly inhibits the GLUT4 contribution (Indinavir), which has been shown in cell lines

[88]. We tried to access this drug to perform such measurements for human adipocytes for the analysis in Paper I, but without success.

INSULIN CONTROL OF ERK SIGNALING

Extracellular signal-regulated kinases 1/2 (ERK), members of the Map kinase family of protein kinases, are multifunctional kinases with activities both in the cytosol and the nucleus of cells. Upon growth factor stimulation, receptor Tyr kinases activate Ras/Raf, and the dual specificity kinases MEK1/2 that phosphorylates ERK at Tyr and Thr residues [89] (**Figure 12**). The phosphorylation of ERK by MEK1/2 occurs in the cytosol of cells [90]. Phosphorylated ERK can translocate to the nucleus to phosphorylate its nuclear targets [91]. One such target is Ets-like gene 1 (Elk1) [92], a nuclear localized transcription factor that is phosphorylated at multiple residues, whereof Ser 383 and Thr 363 have been shown to be important for transcriptional activity [93, 94]. The ERK signaling branch is highly activated by growth factors like the epidermal growth factor (EGF), but is also activated to a smaller extent by insulin [95, 96]. The insulin stimulated phosphorylation of ERK occurs via the proteins Shc [97] and/or IRS1, which both are phosphorylated by the insulin receptor in response to insulin [98] (**Figure 12**).

To be able to further develop the insulin-signaling model and include the ERK pathway, we measured the dynamic response to insulin for ERK phosphorylation (Paper IV). We found a maximal response after 10 minutes and that the signal is back to basal levels at 60 minutes in human adipocytes. In differentiated preadipocytes from human subjects, the first 15 minutes after insulin stimulation have been studied [99]. The authors found a maximal insulin stimulation of ERK phosphorylation at 10 minutes. The same ERK dynamics have also been studied in 3T3-L1 adipocytes that have a higher response to insulin (up to 18-fold) [100]. ERK phosphorylation in this cell line is maximal at 2 minutes. It is also interesting to note that there are differences in the ERK dynamics between different tissues stimulated *in vivo* with insulin in mice [101]. While ERK phosphorylation in muscle tissue continues to increase during 30 minutes, the adipose tissue ERK phosphorylation return to basal levels [101]. In Paper IV, we tested different hypotheses to explain our findings of the insulin stimulated ERK dynamics in human adipocytes.

In 3T3-L1 adipocytes it has been shown that ERK is activated by insulin downstream of PI3K, since the insulin response is reduced with addition of wortmannin, a specific PI3K inhibitor [102]. The same finding is also reported from rat adipocytes stimulated with insulin and inhibited with wortmannin [103-105]. One of the reports, [103], also show that

the Shc pathway is not involved in the activation of ERK in response to insulin in rat adipocytes. Instead the IRS1/PI3K pathway is exclusively used for ERK activation in these adipocytes. This finding is in contrast to findings in some cell lines where the Shc pathway have been shown to be involved in the activation of ERK in response to insulin [106, 107]. We have previously shown in human adipocytes that activation of ERK and Elk1 is downstream of IRS1 since the expression of a mutant IRS inhibits Elk1 response to insulin [108]. However, to be able to fit with all data of phosphorylation of ERK, we included the ERK signaling branch downstream of both IR and IRS1 in our developed insulin signaling model in Paper IV.

Figure 12: Insulin stimulated ERK phosphorylation

The phosphorylation of ERK after insulin stimulation is mediated via the proteins Shc and/or IRS1 that both are associated to the insulin receptor. The subsequent signaling cascade involves Ras, Raf, MEK, and ERK.

Stimulation with different growth factors gives rise to different dynamic behaviors of ERK (reviewed in [109, 110]), and these observations have been analyzed with mathematical models. Brightman and Fell [111] showed that inhibition of a negative feedback in the pathway can explain that ERK phosphorylation in the same cell type can be transient or sustained depending on the growth factor used for stimulation. Also, different cell types stimulated with the same growth factor give rise to different dynamic behaviors [112]. Kiel and Serrano [112] have shown with mathematical models that different feedback-strengths

in different cells could explain these observations. Feedback loops in the ERK signaling network have also been studied to bring light upon other biological phenomena using mathematical modeling approaches [113, 114]. The studied biological phenomena ranges from robustness [115, 116] and oscillations [117-120], to cooperativity [121] and ultrasensitivity [117, 122]. Ultrasensitivity, i.e. that the response to increasing concentrations of growth factor becomes more switch like further down in the signaling cascade [123], was theoretically predicted in ERK signaling by Huang and Ferrell [124] using a mathematical modeling approach. The authors also confirmed this prediction experimentally using extracts of egg cells (xenopus oocytes). Levchenko *et al.* [125] built on to the Huang and Ferrell model to study the effect of addition of scaffolding proteins to the model. Scaffolding proteins bind to several signaling intermediaries and are important in ERK signaling since they can facilitate signal transduction, segregate signaling pathways from crosstalk, and target signals to specific locations in the cell [126, 127]. In the hypothesis testing in Paper IV, we found that scaffolding, but not a simple feedback mechanism, could explain the dynamic ERK phosphorylation pattern in human adipocytes (**Figure 13**).

In Paper IV, we also tested other hypotheses that were compatible with the dynamics of the ERK phosphorylation data (**Figure 13**). One of the hypotheses dealt with the dual phosphorylation of ERK by MEK. This dual phosphorylation process has previously been modeled with so called processive and distributive mechanisms [128-130]. In a distributive phosphorylation mechanism MEK binds to ERK and phosphorylates one of the residues, and then MEK releases single phosphorylated ERK. MEK also binds to single-phosphorylated ERK and phosphorylates the second residue, and then releases dual-phosphorylated ERK. In a processive phosphorylation mechanism, on the other hand, MEK binds and phosphorylates ERK at one residue and then phosphorylates the other residue before dissociation from ERK. Mathematical analysis has revealed that the phosphorylation of ERK is distributive in experimental systems where proteins have been extracted from cells [128, 131]. It has also been shown with mathematical models that addition of crowding agents to the media, to mimic intracellular conditions, make the phosphorylation of ERK processive [132, 133]. However, in cell line experiments combined with mathematical modeling, phosphorylation of ERK was shown to be distributive in response to the cytokine erythropoietin [134]. We tested dual phosphorylation with different mechanisms in Paper IV, and found a simple processive model that could account for the dynamic data from human adipocytes.

Finally, nuclear translocation of ERK could also explain the dynamics of ERK phosphorylation (**Figure 13**). Fujioka *et al.* [135] have studied the details of nuclear translocation of ERK in HeLa cells with experiments and modeling. Using fluorescent

probes, the authors measure interesting parameters of ERK activity, i.e. rate of phosphorylation and dephosphorylation, rate of nuclear import and export, and rate of binding and dissociation with MEK. With these parameters, the authors create an interesting simulation model of ERK signaling and compare the results with independent data. The authors also compare their measured parameter values with values reported in other modeling papers and show major differences in some of the parameters [135]. These differences are probably due to differences in cell type and/or experimental setup and highlight the importance of using data from the same cell type in the development of mathematical models.

Figure 13: Hypothesis testing and a common minimal model

Three hypotheses explain the dynamic ERK phosphorylation data: scaffolding, dual phosphorylation, and nuclear translocation. These hypotheses can all be simplified to the same minimal model.

All the acceptable hypotheses for the dynamics of ERK phosphorylation (Paper IV) shared the same features and could be reduced to a common minimal model (**Figure 13**). The common minimal model includes sequestration of non-phosphorylated ERK and the analysis of the model revealed that more than 40 % of total ERK had to be in this sequestered pool. We studied total ERK protein with immunofluorescence confocal

microscopy to study the location of this sequestration in the adipocyte and found that ERK concentration was high in the nuclear regions. We could, however, not see an insulin effect on this ERK sequestration.

There are also a few famous detailed models of ERK signaling in the literature (reviewed in [136]). Kholodenko *et al.* [137] have developed a mathematical model of the transient EGF response of the upstream signaling intermediaries, and continued to develop the model with more data, also for different temperatures during stimulation [138]. Schoeberl *et al.* [139] also developed an EGF response model. This detailed model with 94 states and 95 parameters, which mostly have been taken from literature, was compared with data for transient phosphorylation of ERK. These two detailed models for ERK signaling were not used to draw conclusions, but to include all known interactions and gather the available knowledge of connections between intermediaries in the ERK signaling network.

Despite all available models of ERK signaling, there are only a few mathematical models for insulin control of the ERK signaling pathway [96, 140, 141]. Borisov *et al.* [96] have studied interactions between insulin- and EGF-stimulated ERK signaling. Aldridge *et al.* [140] have studied insulin and EGF, but also tumor necrosis factor (TNF), involved in inflammation, and focus on the crosstalk between these signaling pathways. Recently, a pure theoretical attempt at modeling the complete insulin signaling network, including ERK, was published [141]. None of these models were based on dynamic data from human adipocytes, and we could not use them in the development of the extended insulin signaling model in Paper IV.

DETAILED MATHEMATICAL MODELS OF THE INSULIN SIGNALING NETWORK

An important first mathematical model of the insulin signaling network, from insulin-IR interactions to GLUT4 translocation, was published more than a decade ago [142]. In this first detailed model of the metabolic part of insulin signaling, Sedaghat *et al.* combined previous models of insulin-IR binding and IR recycling [33, 44] with models of GLUT4 regulation [75, 76] and knowledge about mechanisms and intermediates involved in downstream signaling to control of glucose uptake, i.e. phosphorylation of PI3K and PKB, and GLUT4 translocation [142]. Sedaghat *et al.* used model parameters from the literature and compared the ODE model simulations with steady-state dose-response data from rat adipocytes for some of the signaling intermediaries. However, the lack of time-resolved data is problematic since the authors claim that they describe the dynamics of the insulin

signaling network. Despite the shortcomings of the Sedaghat model, it has been extensively used and also further developed [143-145] and analyzed [146]. Recently, another more theoretical attempt at constructing a detailed model of insulin signaling was published [141]. The authors have formulated simple equations for a sigmoidal activation to simulate dose-response behaviors and compare with data from different sources. Again, in [141] the lack of dynamic data is a limitation of the study.

Figure 14: The detailed insulin signaling model

The insulin signaling model developed in Paper III-IV contains 32 states (IRm, IRm-YP, etc), 41 reactions (v1a-v11b, bold arrows), and 49 parameters. Regular arrows show where activation occurs in the model. Red numbers show the three diabetes parameters: reduced IR concentration, reduced GLUT4 concentration, and reduced mTORC1-derived feedback to IRS1. From Paper IV.

In contrast, a modeling approach of the insulin signaling network in HeLa and C2C12 cell lines has used dynamic data for many signaling intermediaries [59]. The authors focus on the activation of mTORC2 and conclude by hypothesis testing that the activation is not

downstream of PKB, or IRS1, but probably downstream a PI3K that is not effected by the mTORC1-derived feedback to IRS1. The conclusions in [59] depend on the authors confidence in a negative feedback from mTORC1 via S6K to IRS1 phosphorylation, which, at least in human adipocytes, is not important (Paper III). Instead we show that a positive feedback from mTORC1 to phosphorylation of IRS1 at Tyr via Ser 307-phosphorylation of IRS1 is reduced in T2D (Paper III).

With Paper III, we are now able to present a comprehensive ODE model of the insulin signaling network based on extensive experimental data from human adipocytes gathered in our laboratory for several years. The experimental data are both time resolved and dose dependent and gathered for many important signaling intermediaries such as IR, IRS1, PKB, AS160, glucose uptake, and also S6K and S6 as a direct measure of mTORC1 activity. With the presented mathematical model in Paper III we have taken the knowledge of dynamic intracellular insulin signaling to a new level. In Paper IV, we continue to develop this mathematical model and include the ERK signaling pathway and an important signaling contribution from ERK to S6 (**Figure 14**).

EXPERIMENTAL PERTURBATIONS IN MODEL DEVELOPMENT AND VALIDATION

Perturbations of the signaling are important to enhance and complete understanding of the insulin signaling network. Commonly used perturbations at the cellular level are the use of different inhibitors that are specific to one or more protein targets. Rapamycin is a specific inhibitor of mTORC1 that we used as a validation in Paper III, and also to understand the activation of ERK signaling in Paper IV. The effects of rapamycin in human adipocytes are seen in most proteins of the insulin signaling network (**Table 1**), because of the feedback from mTORC1 to phosphorylation of IRS1 at Ser 307. Rapamycin has been reported to lower glucose uptake in human adipocytes [67], although also the contrary has been reported [147]. The previously reported feedback from IRS1 to dephosphorylation of internalized IR (first described in [13]), which is included in the models in Paper III and Paper IV, seems to be important since the phosphorylation of IR is experimentally effected by rapamycin [67]. This reduction is indeed predicted by the models (Paper III).

In renal samples from humans treated orally with rapamycin, the phosphorylation of PKB at Ser 473 was enhanced and the phosphorylation of IRS1 at Tyr was lowered in response to insulin [148]. The oral rapamycin treatment also gave rise to systemic insulin resistance, as measured by enhanced 2 hour plasma glucose and insulin levels [149].

Table 1: Rapamycin effects on insulin stimulated protein phosphorylations

The arrows represent a negative effect ($\downarrow$), no significant effect ($\rightarrow$), and a positive effect ($\uparrow$) of rapamycin on the measured protein phosphorylations in human adipocytes.

Measured protein phosphorylation	Effect of rapamycin	References	Effect of rapamycin in the mathematical models in Paper III-IV
IR (Tyr)	$\downarrow$	[67]	$\downarrow$
IRS1 (Tyr)	$\downarrow \rightarrow$	[66, 67]	$\downarrow$
IRS1 (Ser 307)	$\downarrow$	[66, 67]	$\downarrow$
PKB (Thr 308)	$\rightarrow \uparrow$	[67, 147]	$\downarrow$
PKB (Ser 473)	$\rightarrow \downarrow \uparrow$	Paper III, [67, 147]	$\downarrow$
AS160 (Thr 642)	$\downarrow$	[67]	$\downarrow$
S6K (Thr 421/Ser 424)	$\downarrow$	[67]	$\downarrow$ S6K (Thr 389)
S6 (Ser 235/236)	$\downarrow$	Paper III	$\downarrow$
ERK (Tyr 202/Thr 204)	$\rightarrow$	Paper IV	$\downarrow$

All the *in vitro* effects of rapamycin (**Table 1**) are compatible with our presented detailed mathematical model (Paper III), with the exception for phosphorylation of PKB in differentiated human preadipocytes where rapamycin was reported to increase the phosphorylation of PKB at Ser 473 and Thr 308 [147]. In our measurements of phosphorylation of PKB at Ser 473 (Paper III), we found no inhibition with rapamycin. The detailed mathematical model simulates a minimal decrease in phosphorylation of PKB at Ser 743 with rapamycin, compatible with our measurements (Paper III).

In Paper IV, we measured the effect of rapamycin on insulin-stimulated phosphorylation of ERK, and found no significant effect. These data were not directly compatible with our presented mathematical model where rapamycin slightly decreases the phosphorylation of ERK. However, we could not reject the model since the rapamycin data allow for a decrease in ERK phosphorylation with rapamycin. The slight reduction of ERK with rapamycin in the model is within the uncertainty of the data.

To gain further knowledge about the insulin signaling network, we have also used perturbations with substances other than rapamycin. In Paper IV, we used two different inhibitors of MEK activation (PD184352 and U0126) and found an important contribution of the ERK signaling pathway in the control of the ribosomal protein S6. Both these MEK inhibitors reduced the phosphorylation of S6, but not the phosphorylation of S6K in human adipocytes (Paper IV). We thus concluded that ERK control of S6 likely occurs via ERK-catalyzed phosphorylation of p90-RSK, which is a protein kinase for S6 (reviewed in

[150]). This crosstalk has been suggested in rat adipocytes [151]. We selected the MEK inhibitor PD184352 as it was shown to be a specific MEK inhibitor [152], and supported the conclusion with a different inhibitor of MEK (U0126). We also combined rapamycin with PD184352 and studied the effect on the phosphorylation of S6 (Paper IV). We concluded that the effects of S6K and ERK on the phosphorylation of S6 are additive in human adipocytes.

INSULIN RESISTANCE IN TYPE 2 DIABETES

Type 2 diabetes (T2D) is characterized by impaired beta cell function and insulin resistance in adipose, muscle, and liver tissues. The systemic insulin resistance originates from impaired intracellular insulin signaling in these tissues. The defects of the intracellular insulin signaling have been shown to appear in several branches of insulin signaling and several layers of regulations are altered (reviewed in [98, 153]). Both the activity and levels of insulin signaling proteins are altered in T2D, but there are discrepancies between different studies [153]. These discrepancies could be due to methodology, or to examination of different kinds of cells, or to examination of material from different sub-populations.

Insulin resistance and T2D are tightly linked to obesity, and there is an increasing agreement that an expanding adipose tissue is the origin to the development of T2D [4]. In an experiment supporting this agreement, co-cultures containing differentiated pre-adipocytes obtained from insulin resistant humans and muscle cells obtained from insulin sensitive humans have shown that the insulin resistance can spread from adipocytes to other insulin responding cells [154]. Further, mice modified to have reduced GLUT4 in the adipose tissue, spontaneously develop insulin resistance also in liver and muscle tissues [155]. In a similar experiment where mice were modified to have reduced GLUT4 in the muscle tissue, glucose uptake in the adipose tissue – but not in the liver – was reduced [156].

Adipocytes are resistant to insulin prior to diagnosis of T2D, but the underlying causes to this insulin resistance remain underexplored. Suggested causes are both external factors and intracellular mechanisms. External inflammation, i.e. where macrophages infiltrate the adipose tissue and release cytokines, has been shown to correlate with insulin resistance in the adipose tissue of obese humans [157]. Another possible cause of the insulin resistance is an intracellular response to a nutrient overload. Adipocytes are quite flexible and can increase in size to store more fatty acids as triacylglycerol in the lipid droplet (**Figure 1**). However, they may burst if they increase in size too much since adipocyte size is positively correlated with cell death [158]. Adipocyte size is also positively correlated with insulin resistance and obesity: large adipocytes respond less to insulin than small adipocytes from the same individual [159, 160], and the mean size of adipocytes correlates with insulin

resistance as well as obesity [157, 161]. All in all these data suggest that large and insulin resistant adipocytes in obese individuals may burst due to a nutrient overload. Burst and damaged cells in the tissue attract macrophage infiltration, and indeed macrophages in the adipose tissue of obese humans are clustering around dead adipocytes [158]. The inflammation in the insulin resistant adipose tissue may therefore be a consequence – and not the primary cause – of insulin resistance, although inflammation can aggravate the insulin resistance.

mTORC1, the main regulator of cell growth, is a main hub in the insulin signaling network and has, though feedbacks, the potential to reduce the response to insulin throughout the insulin signaling network [162, 163]. Activation of mTORC1 in response to insulin has been shown to be attenuated in adipocytes from T2D patients [164]. In Paper III, we suggest a mechanism where mTORC1 senses the increased size of the adipocytes in T2D and act to attenuate the responsiveness to insulin in order to reduce further cell growth and accumulation of triacylglycerol. Along the same line, we find a reduced activation of ERK in T2D (Paper IV). This reduced activity of ERK may signal to increase adipogenesis to counteract the insulin resistance, since ERK activity can inhibit cell differentiation via peroxisome proliferator activated receptor γ (PPAR γ) and fibroblast growth factor 21 [165, 166].

MECHANISMS OF INSULIN RESISTANCE IN ADIPOCYTES

The knowledge of mechanisms of insulin resistance in adipocytes has consisted of fragmented and not always compatible data, since different experimental systems have been used to gain the knowledge. The lack of compatible and dynamic data has probably been the main reason for the lack of mathematical models describing insulin resistance in adipocytes. Insulin signaling normally in adipocytes has, however, been modeled to some extent (reviewed in [12]). With this thesis we present, for the first time, a systems understanding for insulin resistance in adipocytes, based on internally consistent data.

To obtain a systems understanding of insulin resistance in adipocytes, we gathered data for critical signaling intermediaries of the insulin signaling pathway (Paper III). We found several differences between insulin signaling in T2D and insulin signaling normally. We found a reduced degree of maximal phosphorylation at steady-state for most signaling intermediaries (**Figure 15**). We also found a reduced sensitivity to insulin, i.e. the concentration of insulin that produces half-maximal response (EC50), such that lower insulin sensitivity results in a right-shift in the dose-response curve to higher concentrations

of insulin and higher EC50 values. This reduced sensitivity does not appear at the level of the insulin receptor, but at the level of IRS1 and most downstream signaling intermediaries (**Figure 15**). In Paper IV, we complemented this knowledge with measurements of ERK signaling in T2D. Also for ERK we found a reduced absolute response to insulin and a reduced sensitivity in adipocytes from T2D patients.

Figure 15: Experimental data for insulin signaling proteins in adipocytes normally and in T2D

Blue (non-diabetic) and red (diabetic) dots and lines show measured protein phosphorylations and glucose uptake in response to different insulin concentrations. A reduced sensitivity to insulin downstream of IR was obtained for most signaling proteins and the glucose uptake. Also the dynamic response to insulin was measured, with lower response at most signaling intermediaries. P denotes phosphorylation, Y phosphorylation at Tyr residues, S phosphorylation at Ser residues, and T denotes phosphorylation at Thr residues. From Paper III.

The detailed model of insulin signaling that we developed in Paper III-IV simulates insulin signaling data from adipocytes both from T2D patients and normal controls (**Figure 16**). The only differences between the normal and T2D versions of the model are three clearly defined diabetes-parameters: reduced IR concentration, reduced GLUT4 concentration, and a reduced mTORC1-IRS1 feedback (**Figure 14**). While the reduced concentrations of IR and GLUT4 have been directly measured [167, 168], the changed feedback was a conclusion from our minimal modeling approach (Paper III). In this approach, we tested different suggested mechanisms to describe both the reduced degree of phosphorylation at all signaling intermediaries, and the reduced sensitivity to insulin downstream of IR. We concluded that suggested insulin resistance mechanisms such as reduced IR and reduced IRS1 concentrations could explain the change in the extent of phosphorylation, but not the reduced sensitivity to insulin. These mechanisms were thus rejected as single explanations. In contrast, a reduced positive feedback or an increased negative feedback to IRS1 could provide a reduced sensitivity to insulin. In the continuous model development we used the known feedback downstream of mTORC1, namely phosphorylation of IRS1 at Ser 307, as a mechanism of alteration in T2D.

Ser/Thr phosphorylations of IRS1 are commonly referred to as mechanisms causing insulin resistance (reviewed in [169-171]). These Ser/Thr phosphorylations have either negative or positive effects on insulin signaling, but negative effects are generally referred to as mechanisms of insulin resistance. For example, animal studies have showed increased phosphorylation of IRS1 at Ser 307 in liver tissue from genetically obese and overfed mice [172]. Such mice models are commonly used to study insulin resistance and T2D. Also, mice with alanine replacement of IRS1 at both Ser 307, Ser 312, and another Ser residue in muscle tissues, were more insulin sensitive than wild-type mice when fed with a high-fat diet [173]. In human adipocytes, on the other hand, phosphorylation of IRS1 at Ser 307 is reduced in T2D (**Figure 15**). This reduced phosphorylation of IRS1 at Ser 307 in T2D adipocytes was reversed in the presence of a Ser protein phosphatase inhibitor (okadaic acid), which also resulted in restored phosphorylation of IRS1 at Tyr [66]. The differences in IRS1 Ser phosphorylations between T2D in human versus obese mice models show the importance of using human primary cells when studying insulin resistance in T2D.

The phosphorylation of PKB in response to insulin was somewhat confusing in adipocytes from T2D patients (Paper III). Neither phosphorylation of PKB at Thr 308 nor at Ser 473 showed a substantially reduced sensitivity to insulin in T2D (**Figure 15**). The reduced sensitivity at the level of IRS1 was thus not transmitted to PKB, but to signaling downstream of PKB (i.e. AS160 and glucose uptake). In the model simulations we allowed for a slight reduction of the sensitivity at PKB in T2D, still compatible with data (Paper III). The magnitude of the dynamic response for phosphorylation of PKB at Thr 308 was

lowered in T2D, but was slightly higher at Ser 473. The model could not capture the elevated phosphorylation of PKB at Ser 473 in T2D (**Figure 16**). Interestingly, reports of an inhibitory feedback from mTORC1 signaling to mTORC2 [174, 175] is a potential explanation to the increased phosphorylation of PKB at Ser 473 in T2D. We have, however, not implemented such a feedback in our model, since these results have not been examined in human adipocytes. There have also been observations of a lower insulin response for phosphorylation of PKB at Ser 473 in adipocytes from T2D patients [176, 177].

Figure 16: Dynamic model simulations of insulin signaling normally and in T2D

The measured protein phosphorylations display different dynamic behaviors at different signaling branches. Signaling through IR-IRS1-PKB displays a fast transient overshoot behavior with an above basal steady state level. The transient behavior is slower and returns to basal levels in ERK-Elk1 signaling. Signaling downstream mTORC1 (S6K-S6) respond even slower without tendency to decrease after 60 minutes of stimulation. The diabetic state (red) has the same dynamic behavior as the normal state (blue) for all signaling proteins, but the magnitude of the response is lower in T2D.

We have previously reported that insulin has similar effects on phosphorylation of ERK in adipocytes from normal and T2D patients [178], and that there is a reduced sensitivity to insulin in the phosphorylation of ERK in T2D [179]. In Paper IV, we examined dynamics of ERK phosphorylation for the normal and T2D states and report a reduced maximal degree of phosphorylation in the T2D state, but without a difference in the fold effect of insulin between normal and diabetic state, similarly to [178]. There are also contradictory findings of an increased basal level of ERK phosphorylation, which is not significantly affected by insulin, in adipocytes from T2D patients [180]. In muscle tissue, ERK signaling is neither decreased nor increased in the diabetic state [181, 182].

LEVELS OF SIGNALING PROTEINS IN TYPE 2 DIABETES

A possible defect in insulin signaling in T2D, which could give rise to the insulin resistance, is reduced levels of some of the proteins in the insulin signaling network. For the early insulin signaling proteins, i.e. IR and IRS1, there are ambiguous results for the protein levels in the normal and T2D states. We have observed a reduction to 55 % of normal protein levels for IR in adipocytes from T2D patients [167]. However, another study reports no differences between normal and T2D protein levels of IR in adipocytes [183]. In insulin binding competition experiments, adipocytes from T2D patients bound less insulin than normal adipocytes for all tested insulin concentrations, and the authors concluded that the decreased binding was caused by a reduced level of IR and not by a change in affinity in T2D adipocytes [184, 185]. Also in muscle tissue from T2D patients there seems to be a reduction of IR protein levels as compared to muscle tissue from normal subjects [182], but the authors provide no quantification of the results.

For IRS1, on the other hand, we have reported that there is no change in protein levels in adipocytes from T2D patients compared with non-diabetic subjects [66, 167], but others report a reduction in IRS1 protein levels [180, 183]. In muscle tissue from T2D patients there was no clear reduction of IRS1 protein levels [182]. In another study, human adipocytes were treated with high insulin and high glucose to make them insulin resistant [186]. After 2 hours of treatment, insulin-stimulated glucose uptake was markedly reduced, but without reduction in IRS1 or GLUT4 protein levels. After 6 or more hours, the IRS1 protein levels were reduced, but the insulin resistance was not caused by the IRS1 reduction since the insulin resistance preceded the IRS1 reduction [186]. The contradictory findings for levels of IR and IRS1 in diabetes can be a result of different handling of cells or individual/sub-population differences. We use our results from human adipocytes [66, 167]

in the creation of the insulin signaling model in Paper III, as they have been obtained in an internally consistent manner.

For the proteins downstream of IRS1 in the insulin signaling network, there are no reported differences in protein levels in cells from normal versus T2D patients in adipose or muscle tissue, except for GLUT4 (**Table 2**). GLUT4 protein levels have been reported to be reduced by 50-80 % in adipocytes [168, 180, 187, 188] and by 30-50 % in muscle tissue [189] from T2D patients. In Paper III and Paper IV, we included reduced IR levels to 55 % of normal as well as reduced GLUT4 levels to 50 % of normal in the T2D state of the models.

Table 2: Reported levels of protein in adipocytes and muscle tissue in type 2 diabetes

The arrows represent less reported level of protein in T2D (↓), or no change between normal and T2D in reported level of protein (→).

Measured protein	Cells/tissue samples	Change of protein level in T2D	References
IR	adipocytes, muscle	↓→	[167, 182, 183]
IRS1	adipocytes, muscle	→↓	[167, 180, 182, 183]
PI3K	muscle	→	[190]
PKB	adipocytes, muscle	→	[176, 177, 190]
mTOR	adipocytes	→	[164]
AS160	muscle	→	[189, 191]
GLUT4	adipocytes, muscle	↓	[168, 180, 187-189]
GLUT1	muscle	→	[189]
S6K	adipocytes	→	[164]
ERK	adipocytes, muscle	→	Paper IV, [180, 182]
Elk1	adipocytes	→	Paper IV

LINKING INTRACELLULAR INSULIN SIGNALING WITH WHOLE-BODY GLUCOSE DYNAMICS

Diseases like T2D are manifested and diagnosed at the whole-body level, but usually arise and are best treated at the intracellular level. A central function of intracellular insulin signaling in muscle and adipose tissue is to increase glucose uptake, which results in lowered levels of blood glucose. To be able to fully understand disease mechanisms of T2D, it is therefore important to relate the results from intracellular studies to the clinically interesting whole-body glucose control. Such relations benefit from mathematical descriptions, and mathematical models exist both at the intracellular insulin signaling level and at the whole-body glucose control level. However, these relations must be quantified in a sound manner; therefore we have developed an approach to link these mathematical models to create multi-level models.

THE DYNAMICS OF GLUCOSE HOMEOSTASIS

Insulin controls plasma levels of glucose by reducing production of glucose in the liver, and by increasing adipose and muscle tissue uptake of glucose (**Figure 17**). The dynamic interplay between glucose and insulin has been extensively analyzed with mathematical models (reviewed in [192]). The Bergman minimal model of glucose-insulin dynamics [193] was developed more than 30 years ago and is still used for applications and in research projects. This minimal model is a simple black-box model with few parameters that can be easily estimated for different populations or individuals. This minimal model is, however, not useful for linking organ or cell models, since the model lacks interpretation of the parameters in terms like “glucose uptake by insulin responding tissues”, “insulin release by pancreatic beta-cells”, etc. In recent years, a novel triple-tracer method [194] for measuring flows of glucose between organs *in vivo* have provided new data for creation of more detailed glucose homeostasis models. The first such model, presented by Dalla Man *et al.* [195], describes the systemic glucose homeostasis in an organ-based manner. The model is based on meal response measurements of glucose and insulin from more than 200 healthy human subjects. An extension of the Dalla Man model has been approved by the

American Food and Drug Administration to replace the use of animals in preclinical trials to certify new insulin pumps [196].

Figure 17: Glucose homeostasis

Glucose is absorbed from the intestine to the plasma after a meal intake. Insulin regulates the glucose release from the liver and the glucose uptake from adipose and muscle tissues to maintain normal glucose plasma levels at all times. From [6].

CREATING A MATHEMATICAL LINK USING DYNAMIC CONSTRAINTS

The relation between *in vitro* experiments with isolated adipocytes on one hand, and the adipocytes in the adipose tissue *in vivo* on the other hand, must be sorted out to be able to link models on these different levels. In Paper I, we developed methods with dynamic constraints from the whole-body level to create a link between our intracellular insulin

signaling models with the Dalla Man model for glucose homeostasis [195]. The dynamic constraints are limitations to the model under development (**Figure 18**). Input constraints are used as input to the intracellular model under development and output constraints are fitted with the output of the model and are thus used as experimental data in the optimization process (**Figure 19**). This use of dynamic constraints in the development of a model secures an agreement between the intracellular model and the input and output of the whole-body level model. In Paper I, we combined insulin signaling data from the intracellular level with dynamic constraints describing input and output of glucose and insulin to the adipose tissue during a meal (**Figure 18**). We searched for parameters for the intracellular model that both fitted our experimental *in vitro* data and the dynamic constraints.

Figure 18: Dynamic constraints are input/output limitations

Interstitial insulin and glucose are input constraints and the glucose uptake is the output constraint to the adipose tissue model under development. Modified from Paper I.

To be able to use the adipose tissue glucose uptake profile as an output constraint, we needed to subdivide the dynamic profile of the glucose uptake in the Dalla Man model [195], because this model describes the total glucose uptake profile. The total glucose uptake profile is primarily the sum of muscle and adipose tissue glucose uptake, but it also contains the glucose uptake of the liver, the kidneys, the brain, and the red blood cells. The glucose uptake of the brain and the red blood cells is not affected by insulin and is thus easily separated from the dynamic profile. The remaining glucose uptake profile was

subdivided with the proportions 20 % to adipose tissue and 80 % to muscle tissue glucose uptake [197]. We ignored the liver glucose uptake since most meal glucose is directly absorbed by the liver before entering the plasma and is thus not accounted for by the Dalla Man model. Meal glucose uptake profiles in the adipose tissue have been measured and calculated with the use of tracers and blood flow measurements [198-201]. Compared to forearm (muscle tissue) glucose uptake, the adipose tissue glucose uptake seems to respond faster after the meal [199]. We used the interstitial insulin profile as provided in the Dalla Man model as one of the input constraints (**Figure 18**). Interstitial insulin is hard to measure, and show values ranging from a few to 50 % of plasma insulin [202-204]. The interstitial insulin profile is important for the conclusions in Paper I, and the link should be further developed when reliable measurements of the adipose tissue interstitial insulin concentration in response to a meal are available.

Figure 19: Modeling approach with dynamic constraints

Dynamic constraints are used as additional experimental data in the estimation of parameters in hypothesis testing with mathematical models. Models must fit both *in vitro* data and dynamic constraints to be acceptable and not rejected. See also **Figure 2**.

MATHEMATICAL MODELS LINKING INSULIN SIGNALING TO GLUCOSE CONTROL

There have been a few modeling projects dealing with the linking of intracellular insulin signaling models to whole-body models of glucose control (reviewed in [10, 12]). Chew *et al.* [205] have linked the Sedaghat insulin signaling model [142] with an early model for whole-body glucose homeostasis [206]. Also, in another effort the Sedaghat model has been linked to models for plasma insulin and glucagon concentrations, insulin and glucagon signaling, and glucose production and utilization [207, 208]. However, these modeling projects are using the Sedaghat model, and the Sedaghat model is based on non-human and non-dynamic data. In Paper I, we developed our own linking approach based on minimal models of insulin signaling and dynamic constraints from the Dalla Man model for glucose control [195].

Figure 20: Linking of insulin signaling models with whole-body models of glucose control

In Paper I, we developed a link between intracellular insulin signaling models and a whole-body glucose control model. In the connection we used minimal insulin signaling models and dynamic constraints from the whole-body level. We also added more details in the insulin signaling model and even more detailed models as sub-models. Modified from Paper I.

In the minimal model analysis in Paper I, we concluded that at least two parameters had to be different between the *in vitro* and *in vivo* conditions in order for the intracellular model to account for the glucose uptake by the adipose tissue in the whole-body model. This conclusion would not have been possible without our two-step approach with an initial minimal model analysis (**Figure 20**). We examined two parameters that possibly are

different between *in vitro* and *in vivo* conditions: handling of cells and blood flow. Handling of isolated adipocytes has been shown to enhance glucose uptake by the cells, i.e. the basal glucose uptake [209]. It is known that insulin increases the adipose tissue blood flow [210], and with higher blood flow, more glucose can reach and be taken up by cells. With these two differences in parameter values between the *in vitro* and *in vivo* conditions, we could fit all data both on the intracellular and the whole-body levels. We could thus continue to fill in the known details in the intracellular insulin signaling models. For instance, we tested to insert a detailed insulin binding sub-model [19] (**Figure 20**). The analysis in Paper I showed that it is possible to link detailed models from different levels using a minimal modeling approach and dynamic constraints. We used the results obtained in Paper I in the linking of the comprehensive insulin signaling model in Paper III with the Dalla Man model of glucose control [195].

With the results of Paper I and III we could also link the insulin resistance simulations in T2D to whole-body glucose control in T2D. This was possible since the Dalla Man model also exist in a T2D version, where glucose and insulin profiles during a meal correspond to the diabetic state [195, 211]. The Dalla Man T2D model was developed using the same equations as the normal Dalla Man model with re-parameterization of all parameters to limited data from T2D subjects. The linking between the Dalla Man T2D model and our developed insulin signaling model for T2D could not provide strong conclusions, but is nevertheless the first example of a multi-level model for insulin resistance in T2D. Simulations of the multi-level model for T2D demonstrated that an increased feedback from mTORC1 to IRS1 Ser 307 increases the dynamic adipose tissue glucose uptake in response to a meal (Paper III).

CONCLUDING REMARKS

In this thesis, I have focused on insulin signaling in human adipocytes; how these cells become resistant to insulin in type 2 diabetes, and how to connect such intracellular data with *in vivo* data from the whole-body level. I also show the potential of mathematical modeling to correctly extract available information from experimental data.

In Paper I, we discover the requirement of additional mechanisms to be able to link intracellular insulin signaling models and data with a whole-body model for glucose control. We suggest that handling of cells and blood flow could be such mechanisms and, with these mechanisms added, we show a successful linking of the models at different levels. This successful linking provides a possibility to simulate clinical responses to intracellular perturbations in the insulin signaling network.

In Paper II, we conclude that internalization of the insulin receptor is important for the transient dynamic profile of phosphorylation of IR and IRS1 in rat adipocytes. We find two different explanations to a counter-intuitive, but apparent, reversed order of peaks in the phosphorylation dynamics using minimal mathematical models. The two found explanations are: i) different responding pools of IR combined with limiting concentration of IRS1, and ii) a negative feedback to IRS1.

In Paper III, we provide a systems understanding of insulin signaling and of insulin resistance. Our main conclusion is that the reduced sensitivity to insulin in adipocytes from T2D subjects can be explained with an attenuated feedback from the protein complex mTORC1 to IRS1. We also present for the first time a connection between a detailed intracellular insulin signaling model and an *in vivo* glucose homeostasis model, where both models are developed using data from normal as well as T2D subjects.

In Paper IV, we build on to the systems understanding of insulin resistance with an inclusion of the ERK signaling branch in the detailed insulin signaling model. We use a minimal modeling approach to show that sequestration of inactive ERK is important for the slow dynamics of ERK phosphorylation, and for its return to basal levels. We also demonstrate a substantial crosstalk between ERK and mTORC1 signaling to control the ribosomal protein S6 and thus protein synthesis.

GENERAL DISCUSSION & FUTURE PERSPECTIVES

We claim to have achieved a first systems understanding of insulin resistance in human adipocytes. What do we mean by a systems understanding? The answer to that question concerns both the way we collect data and our mathematical modeling approach. In the data collection, we use the same cell type from the same laboratory to ensure that our data are compatible. We also obtain both steady state measurements with different insulin inputs, as well as dynamic data where we measure the response to insulin over a period of time. With both these kinds of data for several signaling intermediaries, we believe we capture the dynamic and input dependent behavior of the system. Finally, we obtain data from both non-diabetic and type 2 diabetic subjects – data that are directly comparable – to study the differences both in the magnitude of response and in the sensitivity to insulin for the signaling proteins. The systems understanding of insulin resistance occurs when we use all these data simultaneously in a mathematical modeling approach where the mechanisms of insulin resistance can be identified.

The way we use mathematical modeling has a few unique characteristics: parameter estimation, minimal modeling, and the use of core predictions. We estimate the parameters of the models, i.e. we do not rely on directly measured parameter values which have been tradition in systems biology. There are no reliable measurements of insulin signaling parameters in human adipocytes, but even if there were, these measurements come with an uncertainty that need to be taken into consideration – a fact that often has been neglected by researchers. To be able to estimate parameters, we need to work with as small models as possible, since the optimization problem grows with the square of the number of parameters. We thus use minimal models to find the important mechanisms and fill in more details at a later stage. These details, however, consists only of the things we actually measure, or mechanisms that are essential to explain the experimental data. Another issue with the unknown parameter values is the insufficiency of studying only the single best set of found parameters. Instead, all parameters that provide simulations of the model with statistically acceptable fit with data must be taken into account. The reason for this is that we want to obtain reliable predictions. Different parameters with similar fit with data can namely give totally different predictions for unmeasured variables. We thus need to study all the different parameters to be able to evaluate the uncertainty in the prediction. Predictions with low uncertainty, i.e. core predictions, are interesting predictions since they

can be used to reject or validate the model with the corresponding data. This way of using mathematical modeling we refer to as conclusive modeling since we perform our modeling analyses to be able to draw strong conclusions.

Why use mathematical modeling at all? Can the models provide us with new knowledge? How can we know that the models are correct? These questions address the skepticism that still exists when mathematical models are used in biological and medical research. First, a mathematical model is only “correct” for the data used in the development and testing of that particular model. Data for different cells and different experimental conditions can usually not be simulated with the same model without compensatory parameters. Second, a mathematical model is an approximation and always a simplification of the biological system of interest – which indeed is the very meaning of the concept “model”. In fact, our total knowledge of any biological system will always remain a simplification of the underlying layers of complexity. There will always be new layers of understanding to discover since research is a never-ending process. I believe that our search for answers improves with the use of mathematical models. The reason for improvement is that the use of mathematical modeling forces us to strictly formulate questions and hypotheses. We need to specify what we know and which assumptions we have to introduce. This process brings new light upon the existing data, and often guides us in what new experiments to perform, i.e. the experiments that give the most valuable contribution to the further understanding. A final reason to use mathematical modeling is that the approach provides a tool to simultaneously handle and analyze large data sets, and thus allow for a more correct and complete data interpretation. Unfortunately, the potential of mathematical modeling is yet far from fully realized within biological and medical research.

The mathematical modeling of intracellular insulin signaling and of insulin resistance in adipocytes is not completed with this thesis. For example, the mathematical models developed herein do not account for the important anti-lipolytic effect of insulin in adipocytes, i.e. that insulin counteracts the signaling to release of fatty acids. The anti-lipolytic signaling pathway of insulin and the crosstalk to insulin signaling for control of glucose uptake and protein synthesis may also include potential new mechanisms of insulin resistance. An interesting aspect of anti-lipolysis is that insulin directly interacts with β -adrenergic signaling. Another aspect of the lipolysis control is that fluxes of fatty acids to and from adipocytes contribute to the whole-body energy homeostasis. To be able to handle the effect of these fluxes, we have started a project to extend the whole-body model for glucose control [195] with models of glucose and fatty acid dynamics [212], and of insulin-regulated fatty acid dynamics in response to a meal [213]. A new version of the whole-body model will thus include glucose, insulin, and fatty acid dynamics.

The further development of the multi-level model should also include other insulin and glucose responding organs of importance for glucose homeostasis. Herein, we have contributed with a link to intracellular knowledge from adipocyte insulin signaling, but there are models of liver metabolism [214, 215], pancreatic insulin release [216, 217], and brain control of glucose homeostasis [218, 219], which could potentially further expand the presented multi-level model. Of great interest are, of course, also models of control of skeletal muscle insulin signaling and metabolism. Other future extensions to the multi-level model could include models for other perturbations like exercise [220] or longtime fasting. It would also be interesting to include disease progression of T2D into the model, which also has been described with mathematical models [221].

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REFERENCES

1. Scully, T. (2012) Diabetes in numbers, *Nature*. **485**, S2-3.
2. Herman, M. A. & Kahn, B. B. (2006) Glucose transport and sensing in the maintenance of glucose homeostasis and metabolic harmony, *J Clin Invest*. **116**, 1767-75.
3. Rosen, E. D. & Spiegelman, B. M. (2014) What we talk about when we talk about fat, *Cell*. **156**, 20-44.
4. Smith, U. (2002) 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*. **26**, 897-904.
5. Frayn, K. N. (2002) Adipose tissue as a buffer for daily lipid flux, *Diabetologia*. **45**, 1201-10.
6. Rosen, E. D. & Spiegelman, B. M. (2006) Adipocytes as regulators of energy balance and glucose homeostasis, *Nature*. **444**, 847-53.
7. Kholodenko, B., Yaffe, M. B. & Kolch, W. (2012) Computational approaches for analyzing information flow in biological networks, *Sci Signal*. **5**, re1.
8. Purvis, J. E. & Lahav, G. (2013) Encoding and Decoding Cellular Information through Signaling Dynamics, *Cell*. **152**, 945-56.
9. Janes, K. A. & Lauffenburger, D. A. (2013) Models of signalling networks - what cell biologists can gain from them and give to them, *J Cell Sci*. **126**, 1913-21.
10. Ajmera, I., Swat, M., Laibe, C., Novere, N. L. & Chelliah, V. (2013) The impact of mathematical modeling on the understanding of diabetes and related complications, *CPT: pharmacometrics & systems pharmacology*. **2**, e54.
11. Mendoza, M. C., Er, E. E. & Blenis, J. (2011) The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation, *Trends Biochem Sci*. **36**, 320-8.
12. Nyman, E., Cedersund, G. & Strålfors, P. (2012) Insulin signaling - mathematical modeling comes of age, *Trends Endocrinol Metab*. **23**, 107-15.
13. Brännmark, C., Palmér, R., Glad, S. T., Cedersund, G. & Strålfors, P. (2010) Mass and information feedbacks through receptor endocytosis govern insulin signaling as revealed using a parameter-free modeling framework, *J Biol Chem*. **285**, 20171-9.
14. Cedersund, G. (2012) Conclusions via unique predictions obtained despite unidentifiability--new definitions and a general method, *FEBS J*. **279**, 3513-27.
15. Nyman, E., Strålfors, P. & Cedersund, G. (2012) Multilevel-modeling, core predictions, and the concept of final conclusions in *Biosimulation in Biomedical Research, Health Care and Drug Development* (Mosekilde, E., Sosnovtseva, O. & Rostami-Hogjegan, A., eds) pp. 311-328, Springer Verlag.
16. Sachs, K., Gifford, D., Jaakkola, T., Sorger, P. & Lauffenburger, D. A. (2002) Bayesian network approach to cell signaling pathway modeling, *Sci STKE*. **2002**, pe38.
17. Pe'er, D. (2005) Bayesian network analysis of signaling networks: a primer, *Sci STKE*. **2005**, pl4.

18. Wang, R. S., Saadatpour, A. & Albert, R. (2012) Boolean modeling in systems biology: an overview of methodology and applications, *Physical biology*. **9**, 055001.
19. Kiselyov, V. V., Versteyhe, S., Gauguin, L. & De Meyts, P. (2009) Harmonic oscillator model of the insulin and IGF1 receptors' allosteric binding and activation, *Mol Syst Biol*. **5**, 243.
20. Schmidt, H. & Jirstrand, M. (2006) Systems Biology Toolbox for MATLAB: a computational platform for research in systems biology, *Bioinformatics*. **22**, 514-5.
21. Kirkpatrick, S., Gelatt, C. D., Jr. & Vecchi, M. P. (1983) Optimization by simulated annealing, *Science*. **220**, 671-80.
22. Nelder, J. A. & Mead, R. (1965) A simplex method for function minimization, *The Computer Journal*. **7**, 308-13.
23. Pettersson, T. (2008) *Global optimization models for estimation of descriptive models*, Linköping University.
24. Kreutz, C., Raue, A. & Timmer, J. (2012) Likelihood based observability analysis and confidence intervals for predictions of dynamic models, *BMC Syst Biol*. **6**, 120.
25. Saltiel, A. R. & Kahn, C. R. (2001) Insulin signalling and the regulation of glucose and lipid metabolism, *Nature*. **414**, 799-806.
26. White, M. F. & Kahn, C. R. (1994) The insulin signaling system, *J Biol Chem*. **269**, 1-4.
27. Taniguchi, C. M., Emanuelli, B. & Kahn, C. R. (2006) Critical nodes in signalling pathways: insights into insulin action, *Nat Rev Mol Cell Biol*. **7**, 85-96.
28. Lee, J. & Pilch, P. F. (1994) The insulin receptor: structure, function, and signaling, *Am J Physiol*. **266**, C319-34.
29. Lee, J., Pilch, P. F., Shoelson, S. E. & Scarlata, S. F. (1997) Conformational changes of the insulin receptor upon insulin binding and activation as monitored by fluorescence spectroscopy, *Biochemistry*. **36**, 2701-8.
30. Schäffer, L. (1994) A model for insulin binding to the insulin receptor., *Eur J Biochem*. **221**, 1127-1132.
31. DeMeyts, P. (1994) The structural basis of insulin and insulin-like growth factor-I receptor binding and negative cooperativity, and its relevance to mitogenic versus metabolic signalling., *Diabetologia*. **37**, S135-S148.
32. Marsh, J. W., Westley, J. & Steiner, D. F. (1984) Insulin-receptor interactions. Presence of a positive effect., *J Biol Chem*. **259**, 6641-6649.
33. Wanant, S. & Quon, M. J. (2000) Insulin receptor binding kinetics: modeling and simulation studies., *J Theor Biol*. **205**, 355-364.
34. Corin, R. E. & Donner, D. B. (1982) Insulin receptors convert to a higher affinity state subsequent to hormone binding. A two-state model for the insulin receptor., *J Biol Chem*. **257**, 104-110.
35. Martin, T. J. & May, J. M. (1986) Testing models of insulin binding in rat adipocytes using network thermodynamic computer simulations., *J Recept Res*. **6**, 323-336.
36. Hammond, B. J., Tikerpae, J. & Smith, G. D. (1997) An evaluation of the cross-linking model for the interaction of insulin with its receptor., *Am J Physiol*. **272**, E1136-E1144.
37. Pedersen, O., Hjollund, E., Beck-Nielsen, H., Lindskov, H. O., Sonne, O. & Gliemann, J. (1981) Insulin receptor binding and receptor-mediated insulin degradation in human adipocytes, *Diabetologia*. **20**, 636-41.
38. Olefsky, J. M., Jen, P., Reaven, G. M. & Alto, P. (1974) Insulin binding to isolated human adipocytes, *Diabetes*. **23**, 565-71.

39. Fagerholm, S., Örtengren, U., Karlsson, M., Ruishalme, I. & Strålfors, P. (2009) Rapid insulin-dependent endocytosis of the insulin receptor by caveolae in primary adipocytes, *PLoS One*. **4**, e5985.
40. Marshall, S. (1985) Dual pathways for the intracellular processing of insulin. Relationship between retroendocytosis of intact hormone and the recycling of insulin receptors, *J Biol Chem*. **260**, 13524-31.
41. Marshall, S. (1985) Kinetics of insulin receptor internalization and recycling in adipocytes. Shunting of receptors to a degradative pathway by inhibitors of recycling, *J Biol Chem*. **260**, 4136-44.
42. Cedersund, G., Roll, J., Ulfhielm, E., Danielsson, A., Tidefeldt, H. & Strålfors, P. (2008) Model-based hypothesis testing of key mechanisms in initial phase of insulin signaling., *PLoS Comput Biol*. **4**, e1000096.
43. Hori, S. S., Kurland, I. J. & DiStefano, J. J. (2006) Role of endosomal trafficking dynamics of the regulation of hepatic insulin receptor activity: models for Fao cells., *Ann Biomed Eng*. **34**, 879-892.
44. Quon, M. J. & Campfield, L. A. (1991) A mathematical model and computer simulation study of insulin receptor regulation., *J Theor Biol*. **150**, 59-72.
45. Backer, J. M., Kahn, C. R. & White, M. F. (1989) Tyrosine phosphorylation of the insulin receptor during insulin-stimulated internalization in rat hepatoma cells., *J Biol Chem*. **264**, 1694-1701.
46. Standaert, M. L. & Pollet, R. J. (1984) Equilibrium model for insulin induced receptor down-regulation. Regulation of insulin receptors in differentiated BC3H-1 myocytes., *J Biol Chem*. **259**, 2346-2354.
47. Mangnall, D., Quayle, A. R. & Clark, R. G. (1984) A simple computer model for insulin-receptor interactions and insulin dependent glucose uptake by adipocytes., *Int J Biomed Comput*. **15**, 327-339.
48. Kohanski, R. A. & Lane, M. D. (1983) Binding of insulin to solubilized insulin receptor from human placenta. Evidence for a single class of noninteracting binding sites., *J Biol Chem*. **258**, 7460-7468.
49. Karlsson, M., Thorn, H., Danielsson, A., Stenkula, K. G., Öst, A., Gustavsson, J., Nyström, F. H. & Strålfors, P. (2004) Colocalization of insulin receptor and insulin receptor substrate-1 to caveolae in primary human adipocytes. Cholesterol depletion blocks insulin signalling for metabolic and mitogenic control, *Eur J Biochem*. **271**, 2471-9.
50. Stenkula, K. G., Thorn, H., Franck, N., Hallin, E., Sauma, L., Nystrom, F. H. & Strålfors, P. (2007) Human, but not rat, IRS1 targets to the plasma membrane in both human and rat adipocytes, *Biochem Biophys Res Commun*. **363**, 840-5.
51. Vanhaesebroeck, B. & Alessi, D. R. (2000) The PI3K-PDK1 connection: more than just a road to PKB, *Biochem J*. **346 Pt 3**, 561-76.
52. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R. J., Reese, C. B. & Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B alpha, *Current Biology*. **7**, 261-269.
53. Alessi, D. R. (2001) Discovery of PDK1, one of the missing links in insulin signal transduction. Colworth Medal Lecture, *Biochem Soc Trans*. **29**, 1-14.
54. Sarbassov, D. D., Guertin, D. A., Ali, S. M. & Sabatini, D. M. (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex, *Science*. **307**, 1098-101.
55. Cybulski, N. & Hall, M. N. (2009) TOR complex 2: a signaling pathway of its own, *Trends Biochem Sci*. **34**, 620-7.
56. Manning, B. D. & Cantley, L. C. (2007) AKT/PKB signaling: navigating downstream, *Cell*. **129**, 1261-74.
57. Cybulski, N., Polak, P., Auwerx, J., Ruegg, M. A. & Hall, M. N. (2009) mTOR complex 2 in adipose tissue negatively controls whole-body growth, *Proc Natl Acad Sci U S A*. **106**, 9902-7.
58. Copp, J., Manning, G. & Hunter, T. (2009) TORC-specific phosphorylation of mammalian target of rapamycin (mTOR): phospho-Ser2481 is a marker for intact mTOR signaling complex 2, *Cancer Res*. **69**, 1821-7.

59. Dalle Pezze, P., Sonntag, A. G., Thien, A., Prentzell, M. T., Godel, M., Fischer, S., Neumann-Haefelin, E., Huber, T. B., Baumeister, R., Shanley, D. P. & Thedieck, K. (2012) A dynamic network model of mTOR signaling reveals TSC-independent mTORC2 regulation, *Sci Signal*. **5**, ra25.
60. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. & Hemmings, B. A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1, *The EMBO journal*. **15**, 6541-51.
61. Manning, B. D. (2012) Comment on "A dynamic network model of mTOR signaling reveals TSC-independent mTORC2 regulation": building a model of the mTOR signaling network with a potentially faulty tool, *Sci Signal*. **5**, lc3; author reply lc4.
62. Gual, P., Le Marchand-Brustel, Y. & Tanti, J. F. (2005) Positive and negative regulation of insulin signaling through IRS-1 phosphorylation, *Biochimie*. **87**, 99-109.
63. Kang, S. A., Pacold, M. E., Cervantes, C. L., Lim, D., Lou, H. J., Ottina, K., Gray, N. S., Turk, B. E., Yaffe, M. B. & Sabatini, D. M. (2013) mTORC1 phosphorylation sites encode their sensitivity to starvation and rapamycin, *Science*. **341**, 1236566.
64. Gual, P., Gremaux, T., Gonzalez, T., Le Marchand-Brustel, Y. & Tanti, J. F. (2003) MAP kinases and mTOR mediate insulin-induced phosphorylation of insulin receptor substrate-1 on serine residues 307, 612 and 632, *Diabetologia*. **46**, 1532-42.
65. Carlson, C. J., White, M. F. & Rondinone, C. M. (2004) Mammalian target of rapamycin regulates IRS-1 serine 307 phosphorylation, *Biochem Biophys Res Commun*. **316**, 533-9.
66. Danielsson, A., Öst, A., Nyström, F. H. & Strålfors, P. (2005) Attenuation of insulin-stimulated insulin receptor substrate-1 serine 307 phosphorylation in insulin resistance of type 2 diabetes, *J Biol Chem*. **280**, 34389-92.
67. Pereira, M. J., Palming, J., Rizell, M., Aureliano, M., Carvalho, E., Svensson, M. K. & Eriksson, J. W. (2012) mTOR inhibition with rapamycin causes impaired insulin signalling and glucose uptake in human subcutaneous and omental adipocytes, *Mol Cell Endocrinol*.
68. Rajan, M. R., Fagerholm, S., Jonsson, C., Kjolhede, P., Turkina, M. V. & Stralfors, P. (2013) 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*. **8**, e59725.
69. Wang, C., Mao, X., Wang, L., Liu, M., Wetzel, M. D., Guan, K. L., Dong, L. Q. & Liu, F. (2007) Adiponectin sensitizes insulin signaling by reducing p70 S6 kinase-mediated serine phosphorylation of IRS-1, *J Biol Chem*. **282**, 7991-6.
70. Giraud, J., Leshan, R., Lee, Y. H. & White, M. F. (2004) Nutrient-dependent and insulin-stimulated phosphorylation of insulin receptor substrate-1 on serine 302 correlates with increased insulin signaling, *J Biol Chem*. **279**, 3447-54.
71. Um, S. H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P. R., Kozma, S. C., Auwerx, J. & Thomas, G. (2004) Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity, *Nature*. **431**, 200-5.
72. Aguirre, V., Werner, E. D., Giraud, J., Lee, Y. H., Shoelson, S. E. & White, M. F. (2002) Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action, *J Biol Chem*. **277**, 1531-7.
73. Copps, K. D., Hancer, N. J., Opare-Ado, L., Qiu, W., Walsh, C. & White, M. F. (2010) Irs1 serine 307 promotes insulin sensitivity in mice, *Cell Metab*. **11**, 84-92.
74. Larance, M., Ramm, G. & James, D. E. (2008) The GLUT4 code, *Mol Endocrinol*. **22**, 226-33.
75. Quon, M. J. & Campfield, L. A. (1991) A mathematical model and computer simulation study of insulin sensitive glucose transporter regulation, *J Theor Biol*. **150**, 93-107.
76. Quon, M. J. (1994) Advances in kinetic analysis of insulin-stimulated GLUT-4 translocation in adipose cells, *Am J Physiol*. **266**, E144-50.

77. Holman, G. D., Lo Leggio, L. & Cushman, S. W. (1994) Insulin-stimulated GLUT4 glucose transporter recycling. A problem in membrane protein subcellular trafficking through multiple pools, *J Biol Chem.* **269**, 17516-24.
78. Yeh, J. I., Verhey, K. J. & Birnbaum, M. J. (1995) Kinetic analysis of glucose transporter trafficking in fibroblasts and adipocytes, *Biochemistry.* **34**, 15523-31.
79. Verhey, K. J., Yeh, J. I. & Birnbaum, M. J. (1995) Distinct signals in the GLUT4 glucose transporter for internalization and for targeting to an insulin-responsive compartment, *J Cell Biol.* **130**, 1071-9.
80. Coster, A. C., Govers, R. & James, D. E. (2004) Insulin stimulates the entry of GLUT4 into the endosomal recycling pathway by a quantal mechanism, *Traffic.* **5**, 763-71.
81. Stenkula, K. G., Lizunov, V. A., Cushman, S. W. & Zimmerberg, J. (2010) Insulin controls the spatial distribution of GLUT4 on the cell surface through regulation of its postfusion dispersal, *Cell Metab.* **12**, 250-9.
82. Brewer, P. D., Romenskaia, I., Kanow, M. A. & Mastick, C. C. (2011) Loss of AS160 Akt substrate causes Glut4 protein to accumulate in compartments that are primed for fusion in basal adipocytes, *J Biol Chem.* **286**, 26287-97.
83. Sano, H., Kane, S., Sano, E., Miinea, C. P., Asara, J. M., Lane, W. S., Garner, C. W. & Lienhard, G. E. (2003) Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation, *J Biol Chem.* **278**, 14599-602.
84. Eguez, L., Lee, A., Chavez, J. A., Miinea, C. P., Kane, S., Lienhard, G. E. & McGraw, T. E. (2005) Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein, *Cell Metab.* **2**, 263-72.
85. Kozka, I. J., Clark, A. E., Reckless, J. P., Cushman, S. W., Gould, G. W. & Holman, G. D. (1995) The effects of insulin on the level and activity of the GLUT4 present in human adipose cells, *Diabetologia.* **38**, 661-6.
86. Smith, R. M., Charron, M. J., Shah, N., Lodish, H. F. & Jarett, L. (1991) Immunoelectron microscopic demonstration of insulin-stimulated translocation of glucose transporters to the plasma membrane of isolated rat adipocytes and masking of the carboxyl-terminal epitope of intracellular GLUT4, *Proc Natl Acad Sci U S A.* **88**, 6893-7.
87. Yang, J., Clark, A. E., Kozka, I. J., Cushman, S. W. & Holman, G. D. (1992) Development of an intracellular pool of glucose transporters in 3T3-L1 cells, *J Biol Chem.* **267**, 10393-9.
88. Rudich, A., Konrad, D., Torok, D., Ben-Romano, R., Huang, C., Niu, W., Garg, R. R., Wijesekara, N., Germinario, R. J., Bilan, P. J. & Klip, A. (2003) Indinavir uncovers different contributions of GLUT4 and GLUT1 towards glucose uptake in muscle and fat cells and tissues, *Diabetologia.* **46**, 649-58.
89. Katz, M., Amit, I. & Yarden, Y. (2007) Regulation of MAPKs by growth factors and receptor tyrosine kinases, *Biochim Biophys Acta.* **1773**, 1161-76.
90. Zheng, C. F. & Guan, K. L. (1994) Cytoplasmic localization of the mitogen-activated protein kinase activator MEK, *J Biol Chem.* **269**, 19947-52.
91. Volmat, V., Camps, M., Arkinstall, S., Pouyssegur, J. & Lenormand, P. (2001) The nucleus, a site for signal termination by sequestration and inactivation of p42/p44 MAP kinases, *J Cell Sci.* **114**, 3433-43.
92. Brunet, A., Roux, D., Lenormand, P., Dowd, S., Keyse, S. & Pouyssegur, J. (1999) Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry, *The EMBO journal.* **18**, 664-74.
93. Marais, R., Wynne, J. & Treisman, R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain, *Cell.* **73**, 381-93.
94. Cruzalegui, F. H., Cano, E. & Treisman, R. (1999) ERK activation induces phosphorylation of Elk-1 at multiple S/T-P motifs to high stoichiometry, *Oncogene.* **18**, 7948-57.

95. Young, S. W., Dickens, M. & Tavaré, J. M. (1994) Differentiation of PC12 cells in response to a cAMP analogue is accompanied by activation of mitogen-activated protein kinase. Comparison with the effects of insulin, growth factors and phorbol esters, *FEBS Lett.* **338**, 212-6.
96. Borisov, N., Aksamitiene, E., Kiyatkin, A., Legewie, S., Berkhout, J., Maiwald, T., Kaimachnikov, N. P., Timmer, J., Hoek, J. B. & Kholodenko, B. N. (2009) Systems-level interactions between insulin-EGF networks amplify mitogenic signaling, *Mol Syst Biol.* **5**, 256.
97. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T. & Pelicci, P. G. (1992) A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction, *Cell.* **70**, 93-104.
98. Virkamaki, A., Ueki, K. & Kahn, C. R. (1999) Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance, *J Clin Invest.* **103**, 931-43.
99. Iniguez, G., Ormazabal, P., Lopez, T., Maldonado, D., Avila, A., Roman, R. & Cassorla, F. (2009) IGF-IR/ERK content and response to IGF-I and insulin in adipocytes from small for gestational age children, *Growth Horm IGF Res.* **19**, 256-61.
100. Kayali, A. G., Austin, D. A. & Webster, N. J. (2000) 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.* **49**, 1783-93.
101. Agouni, A., Owen, C., Czopek, A., Mody, N. & Delibegovic, M. (2010) In vivo differential effects of fasting, re-feeding, insulin and insulin stimulation time course on insulin signaling pathway components in peripheral tissues, *Biochem Biophys Res Commun.* **401**, 104-11.
102. Suga, J., Yoshimasa, Y., Yamada, K., Yamamoto, Y., Inoue, G., Okamoto, M., Hayashi, T., Shigemoto, M., Kosaki, A., Kuzuya, H. & Nakao, K. (1997) 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.* **46**, 735-41.
103. Liu, H., Kublaoui, B., Pilch, P. F. & Lee, J. (2000) Insulin activation of mitogen-activated protein (MAP) kinase and Akt is phosphatidylinositol 3-kinase-dependent in rat adipocytes, *Biochem Biophys Res Commun.* **274**, 845-51.
104. Standaert, M. L., Bandyopadhyay, G. & Farese, R. V. (1995) Studies with wortmannin suggest a role for phosphatidylinositol 3-kinase in the activation of glycogen synthase and mitogen-activated protein kinase by insulin in rat adipocytes: comparison of insulin and protein kinase C modulators, *Biochem Biophys Res Commun.* **209**, 1082-8.
105. Molero, J. C., Perez, C., Martinez, C., Villar, M., Andres, A., Fermin, Y. & Carrascosa, J. M. (2002) Activation of MAP kinase by insulin and vanadate in adipocytes from young and old rats, *Mol Cell Endocrinol.* **189**, 77-84.
106. Skolnik, E. Y., Batzer, A., Li, N., Lee, C. H., Lowenstein, E., Mohammadi, M., Margolis, B. & Schlessinger, J. (1993) The function of GRB2 in linking the insulin receptor to Ras signaling pathways, *Science.* **260**, 1953-5.
107. Yamauchi, K. & Pessin, J. E. (1994) Insulin receptor substrate-1 (IRS1) and Shc compete for a limited pool of Grb2 in mediating insulin downstream signaling, *J Biol Chem.* **269**, 31107-14.
108. Stenkula, K. G., Said, L., Karlsson, M., Thorn, H., Kjolhede, P., Gustavsson, J., Soderstrom, M., Stralfors, P. & Nystrom, F. H. (2004) Expression of a mutant IRS inhibits metabolic and mitogenic signalling of insulin in human adipocytes, *Mol Cell Endocrinol.* **221**, 1-8.
109. Marshall, C. J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation, *Cell.* **80**, 179-85.
110. Murphy, L. O. & Blenis, J. (2006) MAPK signal specificity: the right place at the right time, *Trends Biochem Sci.* **31**, 268-75.
111. Brightman, F. A. & Fell, D. A. (2000) Differential feedback regulation of the MAPK cascade underlies the quantitative differences in EGF and NGF signalling in PC12 cells, *FEBS Lett.* **482**, 169-74.

112. Kiel, C. & Serrano, L. (2009) Cell type-specific importance of ras-c-raf complex association rate constants for MAPK signaling, *Sci Signal.* **2**, ra38.
113. Kolch, W., Calder, M. & Gilbert, D. (2005) When kinases meet mathematics: the systems biology of MAPK signalling, *FEBS Lett.* **579**, 1891-5.
114. Cirit, M., Wang, C. C. & Haugh, J. M. (2010) Systematic quantification of negative feedback mechanisms in the extracellular signal-regulated kinase (ERK) signaling network, *J Biol Chem.* **285**, 36736-44.
115. Fritsche-Guenther, R., Witzel, F., Sieber, A., Herr, R., Schmidt, N., Braun, S., Brummer, T., Sers, C. & Bluthgen, N. (2011) Strong negative feedback from Erk to Raf confers robustness to MAPK signalling, *Mol Syst Biol.* **7**, 489.
116. Sarma, U. & Ghosh, I. (2012) Different designs of kinase-phosphatase interactions and phosphatase sequestration shapes the robustness and signal flow in the MAPK cascade, *BMC Syst Biol.* **6**, 82.
117. Kholodenko, B. N. (2000) Negative feedback and ultrasensitivity can bring about oscillations in the mitogen-activated protein kinase cascades, *Eur J Biochem.* **267**, 1583-8.
118. Vera, J., Rath, O., Balsa-Canto, E., Banga, J. R., Kolch, W. & Wolkenhauer, O. (2010) Investigating dynamics of inhibitory and feedback loops in ERK signalling using power-law models, *Mol Biosyst.* **6**, 2174-91.
119. Sarma, U. & Ghosh, I. (2012) Oscillations in MAPK cascade triggered by two distinct designs of coupled positive and negative feedback loops, *BMC research notes.* **5**, 287.
120. Shin, S. Y., Rath, O., Choo, S. M., Fee, F., McFerran, B., Kolch, W. & Cho, K. H. (2009) Positive- and negative-feedback regulations coordinate the dynamic behavior of the Ras-Raf-MEK-ERK signal transduction pathway, *J Cell Sci.* **122**, 425-35.
121. Kamioka, Y., Yasuda, S., Fujita, Y., Aoki, K. & Matsuda, M. (2010) Multiple decisive phosphorylation sites for the negative feedback regulation of SOS1 via ERK, *J Biol Chem.* **285**, 33540-8.
122. Bluthgen, N., Legewie, S., Kielbasa, S. M., Schramme, A., Tchernitsa, O., Keil, J., Solf, A., Vingron, M., Schafer, R., Herzog, H. & Sers, C. (2009) A systems biological approach suggests that transcriptional feedback regulation by dual-specificity phosphatase 6 shapes extracellular signal-related kinase activity in RAS-transformed fibroblasts, *FEBS J.* **276**, 1024-35.
123. Goldbeter, A. & Koshland, D. E., Jr. (1981) An amplified sensitivity arising from covalent modification in biological systems, *Proc Natl Acad Sci U S A.* **78**, 6840-4.
124. Huang, C. Y. & Ferrell, J. E., Jr. (1996) Ultrasensitivity in the mitogen-activated protein kinase cascade, *Proc Natl Acad Sci U S A.* **93**, 10078-83.
125. Levchenko, A., Bruck, J. & Sternberg, P. W. (2000) Scaffold proteins may biphasically affect the levels of mitogen-activated protein kinase signaling and reduce its threshold properties, *Proc Natl Acad Sci U S A.* **97**, 5818-23.
126. Dhanasekaran, D. N., Kashef, K., Lee, C. M., Xu, H. & Reddy, E. P. (2007) Scaffold proteins of MAP-kinase modules, *Oncogene.* **26**, 3185-202.
127. Roskoski, R., Jr. (2012) ERK1/2 MAP kinases: structure, function, and regulation, *Pharmacol Res.* **66**, 105-43.
128. Ferrell, J. E., Jr. & Bhatt, R. R. (1997) Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase, *J Biol Chem.* **272**, 19008-16.
129. Toni, T., Ozaki, Y., Kirk, P., Kuroda, S. & Stumpf, M. P. (2012) Elucidating the in vivo phosphorylation dynamics of the ERK MAP kinase using quantitative proteomics data and Bayesian model selection, *Mol Biosyst.* **8**, 1921-9.

130. Perrett, R. M., Fowkes, R. C., Caunt, C. J., Tsaneva-Atanasova, K., Bowsher, C. G. & McArdle, C. A. (2013) Signaling to Extracellular Signal-regulated Kinase from ErbB1 Kinase and Protein Kinase C: FEEDBACK, HETEROGENEITY, AND GATING, *J Biol Chem.* **288**, 21001-14.
131. Burack, W. R. & Sturgill, T. W. (1997) The activating dual phosphorylation of MAPK by MEK is nonprocessive, *Biochemistry.* **36**, 5929-33.
132. Aoki, K., Takahashi, K., Kaizu, K. & Matsuda, M. (2013) A quantitative model of ERK MAP kinase phosphorylation in crowded media, *Scientific reports.* **3**, 1541.
133. Aoki, K., Yamada, M., Kunida, K., Yasuda, S. & Matsuda, M. (2011) Processive phosphorylation of ERK MAP kinase in mammalian cells, *Proc Natl Acad Sci U S A.* **108**, 12675-80.
134. Schilling, M., Maiwald, T., Hengl, S., Winter, D., Kreutz, C., Kolch, W., Lehmann, W. D., Timmer, J. & Klingmüller, U. (2009) Theoretical and experimental analysis links isoform-specific ERK signalling to cell fate decisions, *Mol Syst Biol.* **5**, 334.
135. Fujioka, A., Terai, K., Itoh, R. E., Aoki, K., Nakamura, T., Kuroda, S., Nishida, E. & Matsuda, M. (2006) Dynamics of the Ras/ERK MAPK cascade as monitored by fluorescent probes, *J Biol Chem.* **281**, 8917-26.
136. Orton, R. J., Sturm, O. E., V, V., Calder, M., Gilbert, D. R. & Kolch, W. (2005) Computational modelling of the receptor-tyrosine-kinase-activated MAPK pathway, *Biochemical Journal.* **392**, 249-261.
137. Kholodenko, B. N., Demin, O. V., Moehren, G. & Hoek, J. B. (1999) Quantification of short term signaling by the epidermal growth factor receptor, *J Biol Chem.* **274**, 30169-81.
138. Moehren, G., Markevich, N., Demin, O., Kiyatkin, A., Goryanin, I., Hoek, J. B. & Kholodenko, B. N. (2002) Temperature dependence of the epidermal growth factor receptor signaling network can be accounted for by a kinetic model, *Biochemistry.* **41**, 306-20.
139. Schoeberl, B., Eichler-Jonsson, C., Gilles, E. D. & Müller, G. (2002) Computational modeling of the dynamics of the MAP kinase cascade activated by surface and internalized EGF receptors, *Nat Biotechnol.* **20**, 370-5.
140. Aldridge, B. B., Saez-Rodriguez, J., Muhlich, J. L., Sorger, P. K. & Lauffenburger, D. A. (2009) Fuzzy logic analysis of kinase pathway crosstalk in TNF/EGF/insulin-induced signaling, *PLoS Comput Biol.* **5**, e1000340.
141. Nijhout, H. F. & Callier, V. (2013) A new mathematical approach for qualitative modeling of the insulin-TOR-MAPK network, *Frontiers in physiology.* **4**, 245.
142. Sedaghat, A., Sherman, A. & Quon, M. (2002) A mathematical model of metabolic insulin signaling pathways., *Am J Physiol.* **283**, E1084-E1101.
143. Smith, G. R. & Shanley, D. P. (2013) Computational modelling of the regulation of Insulin signalling by oxidative stress, *BMC Syst Biol.* **7**, 41.
144. Giri, L., Mutalik, V. K. & Venkatesh, K. V. (2004) A steady state analysis indicates that negative feedback regulation of PTP1B by Akt elicits bistability in insulin-stimulated GLUT4 translocation., *Theor Biol Med Model.* **1**, 2.
145. Vinod, P. K. & Venkatesh, K. V. (2009) Quantification of the effect of amino acids on an integrated mTOR and insulin signaling pathway., *Mol Biosyst.* **5**, 1163-1173.
146. Sumner, T., Shephard, E. & Bogle, I. D. (2012) A methodology for global-sensitivity analysis of time-dependent outputs in systems biology modelling, *J R Soc Interface.* **9**, 2156-66.
147. Tremblay, F., Gagnon, A., Veilleux, A., Sorisky, A. & Marette, A. (2005) Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes, *Endocrinology.* **146**, 1328-37.
148. Di Paolo, S., Teutonico, A., Leogrande, D., Capobianco, C. & Schena, P. F. (2006) Chronic inhibition of mammalian target of rapamycin signaling downregulates insulin receptor substrates 1 and 2

and AKT activation: A crossroad between cancer and diabetes?, *Journal of the American Society of Nephrology : JASN*. **17**, 2236-44.

149. Teutonico, A., Schena, P. F. & Di Paolo, S. (2005) Glucose metabolism in renal transplant recipients: effect of calcineurin inhibitor withdrawal and conversion to sirolimus, *Journal of the American Society of Nephrology : JASN*. **16**, 3128-35.

150. Blenis, J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK., *Proc Natl Acad Sci USA*. **90**, 5889-5892.

151. Lin, T.-A. & Lawrence, J. C. (1994) Activation of ribosomal protein S6 kinases does not increase glycogen synthesis or glucose transport in rat adipocytes., *J Biol Chem*. **269**, 21255-21261.

152. Davies, S. P., Reddy, H., Caivano, M. & Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors, *Biochem J*. **351**, 95-105.

153. Frojdo, S., Vidal, H. & Pirola, L. (2009) Alterations of insulin signaling in type 2 diabetes: a review of the current evidence from humans, *Biochim Biophys Acta*. **1792**, 83-92.

154. Dietze, D., Koenen, M., Rohrig, K., Horikoshi, H., Hauner, H. & Eckel, J. (2002) Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes, *Diabetes*. **51**, 2369-76.

155. Abel, E. D., Peroni, O., Kim, J. K., Kim, Y. B., Boss, O., Hadro, E., Minnemann, T., Shulman, G. I. & Kahn, B. B. (2001) Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver, *Nature*. **409**, 729-33.

156. Zisman, A., Peroni, O. D., Abel, E. D., Michael, M. D., Mauvais-Jarvis, F., Lowell, B. B., Wojtaszewski, J. F., Hirshman, M. F., Virkamaki, A., Goodyear, L. J., Kahn, C. R. & Kahn, B. B. (2000) Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance, *Nat Med*. **6**, 924-8.

157. Kloting, N., Fasshauer, M., Dietrich, A., Kovacs, P., Schon, M. R., Kern, M., Stumvoll, M. & Bluher, M. (2010) Insulin-sensitive obesity, *Am J Physiol Endocrinol Metab*. **299**, E506-15.

158. Cinti, S., Mitchell, G., Barbatelli, G., Murano, I., Ceresi, E., Faloia, E., Wang, S., Fortier, M., Greenberg, A. S. & Obin, M. S. (2005) Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans, *J Lipid Res*. **46**, 2347-55.

159. Franck, N., Stenkula, K. G., Öst, A., Lindstrom, T., Strålfors, P. & Nyström, F. H. (2007) Insulin-induced GLUT4 translocation to the plasma membrane is blunted in large compared with small primary fat cells isolated from the same individual, *Diabetologia*. **50**, 1716-22.

160. Laurencikiene, J., Skurk, T., Kulyte, A., Heden, P., Astrom, G., Sjolín, E., Ryden, M., Hauner, H. & Arner, P. (2011) Regulation of lipolysis in small and large fat cells of the same subject, *J Clin Endocrinol Metab*. **96**, E2045-9.

161. Weyer, C., Foley, J. E., Bogardus, C., Tataranni, P. A. & Pratley, R. E. (2000) Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance, *Diabetologia*. **43**, 1498-506.

162. Hsu, P. P., Kang, S. A., Rameseder, J., Zhang, Y., Ottina, K. A., Lim, D., Peterson, T. R., Choi, Y., Gray, N. S., Yaffe, M. B., Marto, J. A. & Sabatini, D. M. (2011) The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling, *Science*. **332**, 1317-22.

163. Yu, Y., Yoon, S. O., Poulogiannis, G., Yang, Q., Ma, X. M., Villen, J., Kubica, N., Hoffman, G. R., Cantley, L. C., Gygi, S. P. & Blenis, J. (2011) Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling, *Science*. **332**, 1322-6.

164. Öst, A., Svensson, K., Ruishalme, I., Brännmark, C., Franck, N., Krook, H., Sandström, P., Kjölhede, P. & Strålfors, P. (2010) Attenuated mTOR signaling and enhanced autophagy in adipocytes from obese patients with type 2 diabetes, *Mol Med*. **16**, 235-46.

165. Hu, E., Kim, J. B., Sarraf, P. & Spiegelman, B. M. (1996) Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma, *Science*. **274**, 2100-3.

166. Dutchak, P. A., Katafuchi, T., Bookout, A. L., Choi, J. H., Yu, R. T., Mangelsdorf, D. J. & Kliewer, S. A. (2012) Fibroblast growth factor-21 regulates PPARgamma activity and the antidiabetic actions of thiazolidinediones, *Cell*. **148**, 556-67.
167. Danielsson, A., Fagerholm, S., Öst, A., Franck, N., Kjölhede, P., Nyström, F. H. & Strålfors, P. (2009) Short-term overeating induces insulin resistance in fat cells in lean human subjects, *Mol Med*. **15**, 228-34.
168. Maianu, L., Keller, S. R. & Garvey, W. T. (2001) 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, *J Clin Endocrinol Metab*. **86**, 5450-6.
169. Tanti, J. F. & Jager, J. (2009) Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation, *Curr Opin Pharmacol*. **9**, 753-62.
170. Zick, Y. (2005) Ser/Thr phosphorylation of IRS proteins: a molecular basis for insulin resistance, *Sci STKE*. **2005**, pe4.
171. Copps, K. D. & White, M. F. (2012) Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2, *Diabetologia*. **55**, 2565-82.
172. Werner, E. D., Lee, J., Hansen, L., Yuan, M. & Shoelson, S. E. (2004) Insulin resistance due to phosphorylation of insulin receptor substrate-1 at serine 302, *J Biol Chem*. **279**, 35298-305.
173. Morino, K., Neschen, S., Bilz, S., Sono, S., Tsigotis, D., Reznick, R. M., Moore, I., Nagai, Y., Samuel, V., Sebastian, D., White, M., Philbrick, W. & Shulman, G. I. (2008) Muscle-specific IRS-1 Ser->Ala transgenic mice are protected from fat-induced insulin resistance in skeletal muscle, *Diabetes*. **57**, 2644-51.
174. Julien, L. A., Carriere, A., Moreau, J. & Roux, P. P. (2010) mTORC1-Activated S6K1 Phosphorylates Rictor on Threonine 1135 and Regulates mTORC2 Signaling, *Molecular and Cellular Biology*. **30**, 908-921.
175. Liu, P., Gan, W., Inuzuka, H., Lazorchak, A. S., Gao, D., Arojo, O., Liu, D., Wan, L., Zhai, B., Yu, Y., Yuan, M., Kim, B. M., Shaik, S., Menon, S., Gygi, S. P., Lee, T. H., Asara, J. M., Manning, B. D., Blenis, J., Su, B. & Wei, W. (2013) Sin1 phosphorylation impairs mTORC2 complex integrity and inhibits downstream Akt signalling to suppress tumorigenesis, *Nature cell biology*. **15**, 1340-50.
176. Rondinone, C. M., Carvalho, E., Wesslau, C. & Smith, U. P. (1999) Impaired glucose transport and protein kinase B activation by insulin, but not okadaic acid, in adipocytes from subjects with Type II diabetes mellitus, *Diabetologia*. **42**, 819-25.
177. Carvalho, E., Eliasson, B., Wesslau, C. & Smith, U. (2000) Impaired phosphorylation and insulin-stimulated translocation to the plasma membrane of protein kinase B/Akt in adipocytes from Type II diabetic subjects, *Diabetologia*. **43**, 1107-15.
178. Danielsson, A., Öst, A., Lystedt, E., Kjölhede, P., Gustavsson, J., Nyström, F. H. & Strålfors, P. (2005) Insulin resistance in human adipocytes occurs downstream of IRS1 after surgical cell isolation but at the level of phosphorylation of IRS1 in type 2 diabetes, *FEBS J*. **272**, 141-51.
179. Öst, A., Danielsson, A., Liden, M., Eriksson, U., Nyström, F. H. & Strålfors, P. (2007) Retinol-binding protein-4 attenuates insulin-induced phosphorylation of IRS1 and ERK1/2 in primary human adipocytes, *FASEB J*. **21**, 3696-704.
180. Carlson, C. J., Koterski, S., Sciotti, R. J., Pocard, G. B. & Rondinone, C. M. (2003) 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*. **52**, 634-41.
181. Cusi, K., Maezono, K., Osman, A., Pendergrass, M., Patti, M. E., Pratipanawatr, T., DeFronzo, R. A., Kahn, C. R. & Mandarino, L. J. (2000) Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle, *J Clin Invest*. **105**, 311-20.

182. Krook, A., Bjornholm, M., Galuska, D., Jiang, X. J., Fahlman, R., Myers, M. G., Jr., Wallberg-Henriksson, H. & Zierath, J. R. (2000) Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients, *Diabetes*. **49**, 284-92.
183. Rondinone, C. M., Wang, L. M., Lonnroth, P., Wesslau, C., Pierce, J. H. & Smith, U. (1997) 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, *Proc Natl Acad Sci U S A*. **94**, 4171-5.
184. Kolterman, O. G., Gray, R. S., Griffin, J., Burstein, P., Insel, J., Scarlett, J. A. & Olefsky, J. M. (1981) Receptor and postreceptor defects contribute to the insulin resistance in noninsulin-dependent diabetes mellitus, *J Clin Invest*. **68**, 957-69.
185. Freidenberg, G. R., Henry, R. R., Klein, H. H., Reichart, D. R. & Olefsky, J. M. (1987) Decreased kinase activity of insulin receptors from adipocytes of non-insulin-dependent diabetic subjects, *J Clin Invest*. **79**, 240-50.
186. Renstrom, F., Buren, J., Svensson, M. & Eriksson, J. W. (2007) Insulin resistance induced by high glucose and high insulin precedes insulin receptor substrate 1 protein depletion in human adipocytes, *Metabolism*. **56**, 190-8.
187. Hussey, S. E., McGee, S. L., Garnham, A., Wentworth, J. M., Jeukendrup, A. E. & Hargreaves, M. (2011) Exercise training increases adipose tissue GLUT4 expression in patients with type 2 diabetes, *Diabetes Obes Metab*. **13**, 959-62.
188. Garvey, W. T., Maianu, L., Huecksteadt, T. P., Birnbaum, M. J., Molina, J. M. & Ciaraldi, T. P. (1991) Pretranslational suppression of a glucose transporter protein causes insulin resistance in adipocytes from patients with non-insulin-dependent diabetes mellitus and obesity, *J Clin Invest*. **87**, 1072-81.
189. Kampmann, U., Christensen, B., Nielsen, T. S., Pedersen, S. B., Orskov, L., Lund, S., Moller, N. & Jessen, N. (2011) GLUT4 and UBC9 protein expression is reduced in muscle from type 2 diabetic patients with severe insulin resistance, *PLoS One*. **6**, e27854.
190. Kim, Y. B., Nikoulina, S. E., Ciaraldi, T. P., Henry, R. R. & Kahn, B. B. (1999) Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes, *J Clin Invest*. **104**, 733-41.
191. Karlsson, H. K., Zierath, J. R., Kane, S., Krook, A., Lienhard, G. E. & Wallberg-Henriksson, H. (2005) Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of type 2 diabetic subjects, *Diabetes*. **54**, 1692-7.
192. Palumbo, P., Ditlevsen, S., Bertuzzi, A. & De Gaetano, A. (2013) Mathematical modeling of the glucose-insulin system: a review, *Mathematical biosciences*. **244**, 69-81.
193. Bergman, R. N., Ider, Y. Z., Bowden, C. R. & Cobelli, C. (1979) Quantitative estimation of insulin sensitivity, *Am J Physiol*. **236**, E667-77.
194. Basu, R., Di Camillo, B., Toffolo, G., Basu, A., Shah, P., Vella, A., Rizza, R. & Cobelli, C. (2003) Use of a novel triple-tracer approach to assess postprandial glucose metabolism, *Am J Physiol Endocrinol Metab*. **284**, E55-69.
195. Dalla Man, C., Rizza, R. A. & Cobelli, C. (2007) Meal simulation model of the glucose-insulin system, *IEEE Trans Biomed Eng*. **54**, 1740-9.
196. Kovatchev, B. P., Breton, M. D., Dalla Man, C. & Cobelli, C. (2008) In silico model and computer simulation environment approximating the human glucose/insulin utilization, *Food and Drug Administration Master File MAF 1521*.
197. Gerich, J. E. (2000) Physiology of glucose homeostasis, *Diabetes Obes Metab*. **2**, 345-50.
198. McQuaid, S. E., Hodson, L., Neville, M. J., Dennis, A. L., Cheeseman, J., Humphreys, S. M., Ruge, T., Gilbert, M., Fielding, B. A., Frayn, K. N. & Karpe, F. (2011) Downregulation of adipose tissue fatty acid trafficking in obesity: a driver for ectopic fat deposition?, *Diabetes*. **60**, 47-55.

199. Ruge, T., Hodson, L., Cheeseman, J., Dennis, A. L., Fielding, B. A., Humphreys, S. M., Frayn, K. N. & Karpe, F. (2009) Fasted to fed trafficking of Fatty acids in human adipose tissue reveals a novel regulatory step for enhanced fat storage, *J Clin Endocrinol Metab.* **94**, 1781-8.
200. McQuaid, S. E., Humphreys, S. M., Hodson, L., Fielding, B. A., Karpe, F. & Frayn, K. N. (2010) Femoral adipose tissue may accumulate the fat that has been recycled as VLDL and nonesterified fatty acids, *Diabetes.* **59**, 2465-73.
201. Dimitriadis, G., Mitrou, P., Lambadiari, V., Boutati, E., Maratou, E., Koukkou, E., Tzanela, M., Thalassinou, N. & Raptis, S. A. (2006) Glucose and lipid fluxes in the adipose tissue after meal ingestion in hyperthyroidism, *J Clin Endocrinol Metab.* **91**, 1112-8.
202. Mokshagundam, S. P., Peiris, A. N., Stagner, J. I., Gingerich, R. L. & Samols, E. (1996) Interstitial insulin during euglycemic-hyperinsulinemic clamp in obese and lean individuals, *Metabolism.* **45**, 951-6.
203. Bodenlenz, M., Schaupp, L. A., Druml, T., Sommer, R., Wutte, A., Schaller, H. C., Sinner, F., Wach, P. & Pieber, T. R. (2005) Measurement of interstitial insulin in human adipose and muscle tissue under moderate hyperinsulinemia by means of direct interstitial access, *Am J Physiol Endocrinol Metab.* **289**, E296-300.
204. Sandqvist, M., Johanson, E. H., Ahren, B., Axelsen, M., Schmelz, M., Smith, U. & Jansson, P. A. (2006) Postprandial interstitial insulin concentrations in type 2 diabetes relatives, *European journal of clinical investigation.* **36**, 383-8.
205. Chew, Y. H., Shia, Y. L., Lee, C. T., Majid, F. A., Chua, L. S., Sarmidi, M. R. & Aziz, R. A. (2009) Modeling of glucose regulation and insulin-signaling pathways., *Mol Cell Endocrinol.* **303**, 13-24.
206. Cobelli, C. & Mari, A. (1983) Validation of mathematical models of complex endocrine-metabolic systems. A case study on a model of glucose regulation., *Med Biol Eng Comput.* **21**, 390-399.
207. Liu, W. & Tang, F. (2008) Modeling a simplified regulatory system of blood glucose at molecular levels., *J Theor Biol.* **252**, 608-620.
208. Liu, W., Hsin, C. & Tang, F. (2009) A molecular mathematical model of glucose mobilization and uptake., *Math Biosci.* **221**, 121-129.
209. Vega, F. V. & Kono, T. (1979) Sugar transport in fat cells: effects of mechanical agitation, cell-bound insulin, and temperature, *Archives of biochemistry and biophysics.* **192**, 120-7.
210. Iozzo, P., Viljanen, A., Guzzardi, M. A., Laine, H., Honka, M. J., Ferrannini, E. & Nuutila, P. (2012) The interaction of blood flow, insulin, and bradykinin in regulating glucose uptake in lower-body adipose tissue in lean and obese subjects, *J Clin Endocrinol Metab.* **97**, E1192-6.
211. Basu, A., Dalla Man, C., Basu, R., Toffolo, G., Cobelli, C. & Rizza, R. A. (2009) Effects of type 2 diabetes on insulin secretion, insulin action, glucose effectiveness, and postprandial glucose metabolism, *Diabetes Care.* **32**, 866-72.
212. Roy, A. & Parker, R. S. (2006) Dynamic modeling of free fatty acid, glucose, and insulin: an extended "minimal model", *Diabetes technology & therapeutics.* **8**, 617-26.
213. Jelic, K., Hallgreen, C. E. & Colding-Jorgensen, M. (2009) A model of NEFA dynamics with focus on the postprandial state, *Ann Biomed Eng.* **37**, 1897-909.
214. Konig, M., Bulik, S. & Holzhutter, H. G. (2012) Quantifying the contribution of the liver to glucose homeostasis: a detailed kinetic model of human hepatic glucose metabolism, *PLoS Comput Biol.* **8**, e1002577.
215. Konig, M. & Holzhutter, H. G. (2012) Kinetic modeling of human hepatic glucose metabolism in type 2 diabetes mellitus predicts higher risk of hypoglycemic events in rigorous insulin therapy, *J Biol Chem.* **287**, 36978-89.
216. Pedersen, M. G. (2010) A biophysical model of electrical activity in human beta-cells, *Biophys J.* **99**, 3200-7.

217. Pedersen, M. G., Dalla Man, C. & Cobelli, C. (2011) Multiscale modeling of insulin secretion, *IEEE Trans Biomed Eng.* **58**, 3020-3.
218. Gobel, B., Langemann, D., Oltmanns, K. M. & Chung, M. (2010) Compact energy metabolism model: brain controlled energy supply, *J Theor Biol.* **264**, 1214-24.
219. Gobel, B. & Langemann, D. (2011) Systemic investigation of a brain-centered model of the human energy metabolism, *Theory Biosci.* **130**, 5-18.
220. Roy, A. & Parker, R. S. (2007) Dynamic modeling of exercise effects on plasma glucose and insulin levels, *Journal of diabetes science and technology.* **1**, 338-47.
221. De Gaetano, A., Hardy, T., Beck, B., Abu-Raddad, E., Palumbo, P., Bue-Valleskey, J. & Porksen, N. (2008) Mathematical models of diabetes progression, *Am J Physiol Endocrinol Metab.* **295**, E1462-79.

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