DISS. ETH NO. 22332

The role of PKB isoforms in the regulation of pancreatic β-cell mass and function

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES OF ETH ZURICH
(Dr. sc. ETH Zurich)

presented by

MAREN GIL DIETRICH
Master of Science ETH Zurich in Pharmaceutical Sciences
born on 19.09.1983
citizen of Rafz (ZH)

accepted on the recommendation of

Prof. Dr. Matthias Peter, examiner
Prof. Dr. Ernst Hafen, co-examiner
Prof. Dr. med. Daniel Konrad, PhD, co-examiner
Dr. Markus Niessen, co-examiner

2014

Table of contents

Summary

Type 2 diabetes is characterised by chronic hyperglycaemia due to insulin resistance combined with insufficient production of insulin. Capacity of insulin production can increase by expansion of functional pancreatic β-cell mass. Under conditions of overt type 2 diabetes, adaptation of functional β-cell mass is impaired caused by increased rate of apoptosis and decreased proliferation of β-cells.

The adapter protein insulin receptor substrate 2 (IRS2) plays a major role in the regulation of maintenance as well as in compensatory expansion of functional β-cell mass. Mice deficient for *Irs2* are insulin resistant in liver and suffer from diabetes at young age because of β-cell failure. Downstream of IRS2 the protein kinase B (PKB/AKT) is indicated to regulate functional β-cell mass. Three isoforms of PKB are expressed in rodents and in humans: PKBα/AKT1, PKBβ/AKT2, and PKBγ/AKT3. PKBα and PKBβ are required for normal peripheral insulin action but their role in pancreatic β-cells remains enigmatic as indicated by the relatively mild islet phenotype of mice with deficiency for either one of these two isoforms. However, reduced activity of PKB in mouse β-cells results merely in insulin secretion defects with very little β-cell loss. This could be due to redundancy of isoforms but also an alternative explanation has been proposed: Only β-cell compensation could be depending on PKB but not maintenance of islet mass. Furthermore, previously published results indicated that PKBα is the only isoform required for compensation.

The overall goal of my PhD thesis was to clarify the role(s) of PKB isoforms in the regulation of β-cell mass and function. Three specific questions were addressed:

1. Is the function of PKBα and PKBβ conserved in rodent and human islets? In this part proliferation, apoptosis, cell size, and insulin production/secretion were analysed in isolated human islets overexpressing PKBα or PKBβ.
2. Is PKBα required for β-cell compensation under insulin resistance? This was tested in a newly generated mouse model with specific deficiency for *Pkbα* in pancreatic β-cells. Mice were fed a high-fat diet (HFD) to induce insulin resistance.
3. How are PKB isoforms regulated in β-cells by different growth factors? In this part activation of PKB isoforms at the two regulated phosphorylation sites was studied in β-cells after stimulation with insulin and/or IGF1.

Results obtained show that functions of PKBα and PKBβ are conserved between human and rodent islets. Both isoforms have specific but also redundant functions. Only PKBα increased significantly β-cell proliferation and protected from apoptosis. β-cell size was regulated by PKBα and PKBβ. Insulin secretion and production were not affected after overexpression of PKBα or PKBβ.

Furthermore, results strongly indicate that *Pkbα* is required for β-cell compensation as mice deficient for *Pkbα* (*βpkbaKO*) developed age-dependent glucose intolerance and HFD accelerated the onset of deteriorated glucose tolerance. Plasma insulin levels from HFD-fed *βpkbaKO* mice were decreased compared to control littermates, indicating failure of β-cell compensation. A first preliminary analysis of pancreas morphology revealed decreased β-cell area relative to total pancreas area in *βpkbaKO* mice compared to control littermates.

SUMMARY

Regulation of PKB isoforms by insulin and IGF1 was found to be complex. Differences were observed with respect to the overall extent of activation but also with respect to which isoform and which phosphorylation site.

PKB isoforms are not equivalent in pancreatic islets as they show differential regulation and function. PKBα appears to be the most important isoform with the greatest potential to regulate islet mass. This finding should be taken into account in therapeutic approaches to improve islet function in diabetes, as isoform-specific intervention might minimise unwanted systemic side effects. Furthermore, current standard methods to detect activation of PKB in β-cells do not allow to distinguish between isoforms. Their validity should therefore be reconsidered.

Zusammenfassung

Periphere Insulinresistenz in Kombination mit ungenügender Insulinproduktion durch die pankreatischen β-Zellen führt zu chronischer Hyperglykämie, das Hauptsymptom der Krankheit Typ 2 Diabetes. Ein gesunder Organismus kann die Kapazität der Insulinproduktion durch erhöhte Zellteilung, Reduzierung des Zelltodes, sowie durch ein verstärktes β-Zellwachstum steigern. In der Pathogenese des Typ 2 Diabetes ist diese Adaptation der β-Zellmasse an den erhöhten Insulinbedarf gestört.

Das Adapterprotein Insulin Rezeptor Substrat 2 (IRS2) spielt sowohl in der Regulation der kompensatorischen Zunahme als auch in der Erhaltung der β-Zellmasse eine wichtige Rolle. Mäuse mit einem Defekt im *Irs2* Gen sind insulinresistent in der Leber und entwickeln schon in jungen Jahren einen manifesten Typ 2 Diabetes aufgrund eines Versagens ihrer β-Zellen. Die Proteinkinase B (PKB) agiert unterhalb von IRS2 im Signalweg und sollte deshalb eine prominente Rolle in der Regulation der β-Zellmasse spielen. Drei verschiedene PKB Isoformen werden in Nager und Menschen exprimiert: PKBα/AKT1, PKBβ/AKT2 und PKBγ/AKT3. Es ist bereits bekannt, dass PKBα und PKBβ eine wichtige Rolle in der peripheren Insulinwirkung spielen, jedoch ist ihre Funktion in der Regulation der β-Zellmasse noch unklar. Weder ein Verlust von PKBα noch von PKBβ führt zu starker Beeinträchtigung der β-Zellmasse. Auch führt eine Reduktion der PKB Aktivität um 80% in Mäusen zwar zu einer gestörten Insulinsekretion, jedoch weisen diese Mäuse nur einen kleinen β-Zellverlust auf. Partielle Redundanz in den Funktionen der einzelnen Isoformen wäre eine mögliche Erklärung für diese Beobachtungen. Jedoch wurde bereits früher eine alternative Hypothese aufgestellt welche postuliert, dass die Erhaltung der β-Zellmasse unabhängig von PKB reguliert wird, diese jedoch eine wichtige Rolle in der kompensatorischen Zunahme der β-Zellmasse spielt. Des Weiteren weisen bereits publizierte Daten darauf hin, dass vorallem PKBα eine wichtige Rolle in der Regulation der β-Zellmasse spielt.

Das Hauptziel meiner Arbeit war die Funktionen der einzelnen PKB Isoformen in der Regulation der β-Zellmasse zu entschlüsseln. Dazu wurden 3 Fragestellungen definiert:

1. Sind die Funktionen von PKBα und PKBβ in pankreatischen β-Zellen zwischen Nager und Menschen evolutionär konserviert? Um diese Frage zu beantworten, wurden PKBα und PKBβ in isolierten menschlichen Inseln überexprimiert und anschliessend wurden das Ausmass der Apoptose, der Proliferation sowie das β-Zellwachstum mit dem von nicht transfizierten Inseln verglichen.
2. Ist PKBα verantwortlich für die kompensatorische Zunahme der β-Zellmasse unter peripherer Insulinresistenz? Um diese Frage zu beantworten wurde ein neues Mausmodell generiert, mit einem spezifischen Knockout für das *Pkbα* Gen in β-Zellen (*βpkbaKO*). Um eine Insulinresistenz auszulösen, wurden die Mäuse mit einer fett-und kalorienreichen (HFD) Diät gefüttert.
3. Wie werden die PKB isoformen von verschiedenen Wachstumsfaktoren reguliert? Um den Aktivierungsgrad der einzelnen Isoformen zu bestimmen wurden in diesem Projekt β-Zellen mit Insulin und/oder IGF1 stimuliert und anschliessend wurde das Ausmass der Phosphorylierung der PKB Isoformen bestimmt.

Unsere Daten zeigen, dass die Funktionen der Isoformen evolutionär konserviert sind in Inseln von Nager und Menschen. Sie haben sowohl spezifische aber auch redundante Funktionen. Nur PKBα induziert die

Proliferation der β-Zellen und schützt diese vor Apoptose. Hingegen können beide Isoformen das β-Zellwachstum stimulieren. Die Insulinproduktion und Sekretion hat sich weder nach Überexpression von PKBα noch von PKBβ verändert.

Des Weiteren weisen unsere Daten daraufhin, dass PKBα verantwortlich ist für die kompensatorische Zunahme der β-Zellmasse, da *βpkbaKO* Mäuse in fortgeschrittenem Alter eine ausgeprägte Glucose Intoleranz aufweisen. Der Einsatz von HFD verschiebt das Auftreten der Glucose Intoleranz auf einen früheren Zeitpunkt. Tiefere Insulinkonzentrationen im Plasma von *βpkbaKO* Mäusen verglichen mit der Kontrollgruppe weisen auf ein Versagen der kompensatorischen Zunahme der β-Zellmasse hin. Erste Analysen der Pankreasmorphologie zeigen eine verminderte Anzahl β-Zellen in *βpkbaKO* Mäusen im Vergleich zu den Kontrollmäusen.

Die Regulation der PKB Isoformen nach Stimulation mit Insulin und IGF1 zeigt sich in unseren Experimenten als komplex. Es wurden sowohl Unterschiede im Ausmass der Aktivierung der totalen PKB gefunden als auch Unterschiede in der Isoform-spezifischen Aktivierung jeweils an beiden Phosphorylierungsseiten (Ser473 und Thr308).

PKB Isoformen werden unterschiedlich reguliert und unterscheiden sich in ihrer Funktion in pankreatischen Inseln. PKBα ist vermutlich die wichtigste Isoform in Inseln, da sie das grösste Potential aufweist die Inselmasse zu regulieren. Unsere Daten könnten dazu beitragen, neue gezieltere therapeutische Ansätze für eine Verbesserung der β-Zellfunktion in der Pathogenese von Typ 2 Diabetes zu entwickeln. Isoform-spezifische Interventionen können unerwünschte Nebenwirkungen vermindern. Die im Moment angewandten Standartmethoden welche die Ermittlung der PKB Aktivierung ermöglichen unterscheiden meistens nicht zwischen den einzelnen Isoformen. Dieser Ansatz sollte in der Zukunft überdacht und angepasst werden.

1. Introduction

1.1 Pancreatic islets of Langerhans

The pancreatic islets of Langerhans represent the endocrine part of the pancreas. In humans, they have a typical diameter between 30 - 300 µm, and are dispersed trough the exocrine part of the pancreas. The endocrine compartment comprises about 2-3% of the pancreas, whereas the exocrine compartment, responsible for the production and release of enzymes into the duodenum that are important for the digestion, represents the main part [171]. Islets are highly-vascularized "mini-organs" [29, 151] composed of various endocrine cell types: β (insulin-producing), α (glucagon-producing), δ (somatostatin-producing), PP (pancreatic peptide-producing), and ε (ghrelin-producing) cells [252]. The main function of pancreatic islets is to control blood glucose homeostasis [252]. Rodent and human islets comprise the same endocrine cell types but display structural variations. Islets from rodents show a central core of β-cells within a layer of other endocrine cells, mostly α-cells. 60-80% of all endocrine cells in rodents are β-cells and 15-20% are α-cells. The size can vary between 10 and 10'000 cells per individual islet.
The constitution of human islets can vary significantly. The number of β-cells in human islets is typically in a range between 28-75%, the number of α-cells varies between 10-65%, and the number of δ-cells varies between 1-22%. Smaller islets typically contain greater numbers of β-cells and fewer α-cells compared to bigger islets [140].
Blood vessels and other non-endocrine cells (nerve fibers, macrophages, dendritic cells, and pericytes) are embedded in to the connective tissue between the islet cell trabeculae [56]. The density of vascularization is around 5 times higher in islets than in the exocrine part of the pancreas [17, 307]. Blood vessels play a pivotal role in the regulation of glucose homeostasis, because of high O_2 consumption of β-cells and the necessity to efficiently distribute released islet hormones via the blood stream. The pancreatic veins enter into the portal venous system. The parasympathetic system stimulates and sympathetic intervention inhibits insulin secretion in islets, respectively [9]

1.2 The regulation of glucose homeostasis

D-Glucose (in the remainder called glucose) is beside of free fatty acids (FFAs) a crucial energy source for the cells of our body. Throughout the day, plasma glucose is maintained between 4 and 9 mM. Fluctuations arise due to variations in uptake (food-intake) and usage (physical exercise or prolonged fasting) of glucose [91], and only tight control trough the antagonistic hormones insulin and glucagon produced in pancreatic islets that lower or increase blood glucose, respectively, avoids hyper- or hypoglycaemia [91, 162]. Hyperglycaemia causes micro and macro vascular damages and accelerates the onset and progress of diabetes mellitus [1, 2], while hypoglycaemia can result in brain injuries, diabetic coma or death [91].

1.2.1 Insulin and glucagon maintain glucose homeostasis

1.2.1.1 Insulin

Insulin is a peptide hormone. Human insulin contains 51 amino acids and is produced and secreted from β-cells. It has strong hypoglycaemic action [20, 27]. Biosynthesis of insulin starts in the endoplasmic reticulum (ER). Insulin mRNA is translated into the precursor prepro-insulin, that is biologically inactive and composed of three linked domains/peptides: A, B and C. Cleavage of the C peptide (31 amino acids) in the Golgi apparatus activates insulin [203, 221, 250, 251, 258]. Insulin is stored complexed to zinc in 9-13'000 secretory vesicles in the cytoplasm [57, 201]. Besides FFAs, keto acids and amino acids, the main stimulus of insulin secretion is glucose. Glucose enters constitutively into β-cells via glucose-transporter (GLUT2) [202].

The phosphorylation of glucose to glucose-6-phosphate by glucokinase is the initial step of glycolysis, which in turn generates nicotinamidadenindinukleotid (NADH), adenosine triphosphate (ATP) and pyruvate. Further break down of pyruvate within the mitochondria of β-cells generates more ATP and an increase of ATP/ ADP (adenosine diphosphate) ratio results in the closure of ATP-dependent-K^+-channels (K_{ATP} channels) leading to membrane depolarization of β-cells. Subsequently, L-type voltage-dependent Ca^{2+}-channels (VDCC) open and Ca^{2+} influx increases cytosolic Ca^{2+} concentration. Ca^{2+}-induced fusion of vesicles to the plasma membrane releases insulin. Insulin secretion is biphasic when extracellular glucose concentration rises fast from 3mmol/L to 16.7-22.2 mmol/L and is kept constant [98]. The first phase of insulin secretion depends on secretion from a readily releasable pool (RRP) of vesicles, mostly docked already to the plasma membrane, whereas for the second phase of insulin secretion a reserve pool (RP) is mobilised and de novo synthesis of insulin is involved [10, 34, 221]. The first phase of insulin secretion lasts 5-6 minutes, whereas the second phase of insulin lasts up to 60 minutes in which the insulin release increases gradually [98].

1.2.1.1.1 Insulin decreases blood glucose levels and restocks fuel reserves

Insulin is the main regulator of blood glucose homeostasis in mammals [31]. Important insulin-sensitive targets with regard to its metabolic action are liver, muscle, and adipose tissue. These tissues form a finely tuned system, crucial for the regulation of blood glucose homeostasis. Insulin increases glucose uptake into muscle and adipose cells, stops glucose production in liver, mediates the storage of glucose as glycogen, induces the synthesis of proteins and fatty acids and also mediates the conversion of glucose to fatty acids. Furthermore, insulin inhibits breakdown of proteins into amino acids and it suppresses the breakdown of triglycerides (TGs) into fatty acids in adipose tissue. In addition, insulin regulates many different targets involved in proliferation and growth, and targets responsible for numerous enzymatic and structural processes.

The liver responds first to fluctuations in plasma insulin levels because blood from pancreas exits into the portal venous system that disembogues firstly into liver. Before the portal venous blood discharges in the liver, the concentration of insulin is 5 fold higher compared to the concentration after passage through the liver. Insulin mediates the breakdown of glucose and promotes the synthesis of glycogen. The conversion

of glucose to glycogen, mostly in liver and muscle, in postprandial periods allows mammals to store carbohydrates for later use. Insulin diminishes also gluconeogenesis in liver. In addition, insulin promotes lipid synthesis and inhibits the oxidation of fatty acids in the liver. TGs are stored in lipid droplets in the liver or are exported as very-low-density lipoprotein (VLDL) particles to muscle and adipocytes, which take them up for storage or to oxidize them for fuel. In addition, insulin stimulates protein synthesis and simultaneously inhibits proteolysis [31]. Insulin decreases circulating glucose levels mainly by regulating a glucose transporter 4 (GLUT4) that is specifically expressed in muscle and adipose tissue. It induces the translocation of GLUT4-containing vesicles to the plasma membrane thereby allowing more glucose to enter [116, 139]. The insulin-stimulated GLUT4 translocation is regulated via activation of three pathways. Firstly, insulin-dependent activation of proteinkinase B (PKB) inhibits its downstream target AKT substrate 160 (AS160) that facilitates docking of vesicles to plasma membrane. Second, insulin promotes GLUT4 translocation via activation of proteinkinase C (PKC) and third, directly via promoting the CAP-Cbl-Tc10 pathway [83].

Most from adipocytes absorbed glucose is converted to TGs. Insulin also promotes the incorporation of fatty acids from chylomicrons and VLDLs into adipocytes [31]. Chylomicrons are lipoprotein particles consisting of TGs, cholesterol, proteins and phospholipids. For that, insulin stimulates the synthesis of lipoprotein lipase (LPL) that is exported out from adipocytes to the surface of endothelial cells where it cleaves TGs from chylomicrons and VLDLs into glycerol and fatty acids. The absorbed fatty acids are converted into TGs and stored in adipocytes [31]. Insulin regulates appetite and peripheral glucose utilisation via the brain [100, 211].

1.2.1.2 Glucagon

Glucagon increases blood glucose. It is formed from pro-glucagon (160 amino acids) and contains 29 amino acids in its mature form. Pro-glucagon mRNA is found in pancreatic α-cells, in hindbrain neurons and in L-cells of the intestinal mucosa [31, 99]. Cleavage of pro-glucagon to glucagon by convertase 2, releases in addition to glucagon also glicentin and the incretins glucagon-like peptides 1/2 (GLP1/2) [22, 274]. Incretins are gastrointestinal hormones, released after a meal. They promote insulin secretion and production. Additionally, they improve insulin sensitivity in periphery. Glucagon secretion is inhibited and gastric motility is decreased. FFAs and glucose stimulate GLP1 and GLP2 secretion from L-cells, whereas proteins have no effect. [160, 184]. GLP2 is additionally known to induce and reduce intestinal proliferation and apoptosis, respectively [67, 268]. Effects of GLP2 on glucose transport and gastric emptying are not fully understood yet [31, 65].

In α-cells, glucagon is stored in secretory granules [21]. The main stimulus for glucagon secretion is hypoglycaemia, but amino acids or the autonomic nervous system can stimulate the secretion as well. Sympathetic and parasympathetic interventions increase Ca^{2+} concentration in α-cells and thus stimulate glucagon secretion. Other islet cells (mainly β-cells) are thought to inhibit glucagon secretion via paracrine and endocrine signalling. Insulin and γ-aminobutyric acid (GABA) inhibit glucagon secretion via hyperpolarisation of the α-cell membrane [19, 99, 194].

1.2.1.2.1 Glucagon promotes hepatic glucose production

The secretion of glucagon is tightly regulated by plasma glucose concentration and is low under normal conditions in healthy individuals. Under conditions where blood glucose levels are low, the glucagon concentration rises quickly and vice versa glucagon concentration in circulation declines rapidly when blood glucose level rises [31, 51]. The primary target of glucagon action is the liver. First, glucagon stimulates hepatic glucose production and inhibits glycogen synthesis. Second, it promotes the glycogen breakdown and activates gluconeogenesis. Further, glycolysis and carbohydrate oxidation are suppressed. Third, oxidation of fat is enhanced after glucagon secretion. All glucagon actions ensure sufficient plasma glucose levels for covering the requirements of glucose-consuming organs during fasting periods or phases of high-energy consumption (physical activity). Extrahepatic effects have also been described: These include the promotion of proteolysis in muscle and lipolysis in adipose tissue. However, these effects only occur under high glucagon concentrations and might play a minor role in the acute regulation of glucose homeostasis under physiological conditions [31, 50].
Glucagon acts via activation of its G-protein coupled transmembrane receptor and regulates two pathways: First, activation of adenylyl cyclase increases intracellular 3, 5'-cyclic adenosine monophosphate (cAMP) concentrations. Rising cAMP concentration in cytosol activates PKA. PKA phosphorylates the transcription factor cAMP response element (CREB) at serine residue, which in turn binds to his coactivator CREB binding protein (CBP). This leads to transcription of genes required for various pathways involved in the regulation of hyperglycaemic actions of glucagon, e.g conversion of glycogen to glucose in muscle or secretion of glucagon from α-cells [11, 304]. Glucagon regulates various other processes independent from regulation of glucose homeostasis often via activation of phospholipase C (PLC). PLC mediates among others cellular processes in hepatocytes such as growth and proliferation [279]. Receptors activated by glucagon are expressed in various tissues. High expressions are found of course in liver, but also in kidney and α-and β-cells [15] .The role of α-cells and glucagon in the pathogenesis of diabetes type 2 (T2D) is not fully elucidated yet. Hyperglucagonaemia can be found in patients with T2D stimulating hepatic glucose production and contributing therefore to chronic hyperglycaemia. Additionally, decline of β-cell mass lead to increased glucagon levels relative to insulin and therefore to impaired paracrine and endocrine signalling, which in turn might contribute to pathogenesis of T2D. However, detailed underlying mechanisms are still unclear [94].

1.2.2 β-cell mass can adapt to meet the demand for insulin production

Although once believed, an organism is not born with all of the β-cells it will ever have. After birth and during adult live the mass of pancreatic β-cells is not static but can increase or decrease [6]. Existing β-cells contribute to the slowly renewing population with a constant but low rate of proliferation and apoptosis. Islet mass expansion over the lifespan of an organism prevents diabetes in the face of age-related increase in body mass and insulin resistance [191].
Pancreatic β-cell mass can adapt in accordance to demand for insulin [45, 217]. During obesity, functional β-cell mass can increase via enhanced proliferation, β-cell growth, neogenesis,

transdifferentation and reduced apoptosis and thus preventing the development of T2D [217, 220]. Transdifferentation of α-cells leads to newly formed β-cells, whereas during neogenesis β-cells arise from ductal cell precursors [92]. Many positive regulators of β-cell mass are known. Important factors are insulin, insulin-like growth factor 1 (IGF1), FFAs, glucose, and GLP1 [66, 248, 249, 275].
During pregnancy, pancreatic β-cell mass can increase extensively via stimulation by placental lactogens (PLs), prolactin (Prl) and growth hormons (GHs) and thus preventing gestational diabetes [36, 37, 135, 205]. Such compensatory changes are reversible. Within 10 days after birth, β-cell mass in the mother decreases via enhanced β-cell apoptosis, decreased proliferation and β-cell atrophy [233].

1.3 Insulin signalling

1.3.1 Crosstalk between insulin-like growth factor- (IGFR) and insulin receptor (IR) signalling

Insulin is responsible for the fine-tuning of various biological processes, such as cellular growth, proliferation, glucose metabolism, apoptosis and cell differentiation [72]. Nearly every cell in vertebrates expresses IRs but classic insulin-responsive cells with high expression of IRs are hepatocytes, adipocytes and muscle cells with up to 200'000 IRs [283, 293]. Defective insulin signalling pathway leads to metabolic disorders like glucose intolerance, hypertension, dyslipidaemia and T2D [284]. Insulin and IGF1/2, key regulators of embryonic growth and cell differentiation, share the highly conserved insulin signalling pathway [70]. Insulin and IGF1/2 share about 40-80% sequence homology. The IGFR and IR share 85% amino acid identity in the intracellular kinase domain and 100% conservation in the ATP pocket [143, 282]. However, each ligand shows significantly higher affinity to his affiliated receptor [246, 282, 284]. Two different splice variants of IR exist: IRa and IRb [285]. IRb is known to mediate metabolic signalling pathways, whereas IRa activates mitogenic signalling pathways [246]. Insulin binds with affinity to IRa, IRb, and IGF1R, but interacts also with the hybrid receptors IGF1R/IRa and IGF1R/IRb. IGF1 activates IGF1R and the hybrid receptors IGF1R/IRa or IGF1R/IRb. IGF2 binds to IRa, IGF1R, or to IGF1R/IRa. IR and IGFR consist of two extracellular α-subunits linked to each other and to two trans-membrane β-subunits via disulfide bridges [73]. Upon ligand binding to the α-subunits, activation of an intrinsic tyrosine kinase activity on the β-subunit results in autophosphorylation on several tyrosines of the receptor. These phosphotyrosines act as docking sites for downstream substrates of the IR/IGFR. Important substrates are the so-called insulin receptor substrate (IRS) and Src homology domain-containing (SHC) proteins. These adapter proteins are phosphorylated themselves upon binding to the activated receptor and this initiates various cascades of complex cellular signalling responses [83] (Figure 1). IRS and SHC proteins do not have enzymatic activity but they provide docking sites for proteins containing Src homology 2 (SH2) domains like the regulatory subunit (p85) of phosphatidyl inositol 3-kinase (PI3K) or other adapter proteins like growth factor receptor-bound protein 2 (Grb2) [283]. At least three main pathways can be distinguished downstream of IR/IGFR: the PI3K - PKB pathway, regulating metabolic actions and cellular growth and the Ras-mitogen-activated protein kinase (MAPK) pathway, mainly responsible for mitogenic actions in cells [261]. The third pathway called (CAP)-Cbl-Tc10 is

activated via Cbl-associated protein (CAP) and contributes to the regulation of GLUT4 translocation (not shown) [210].

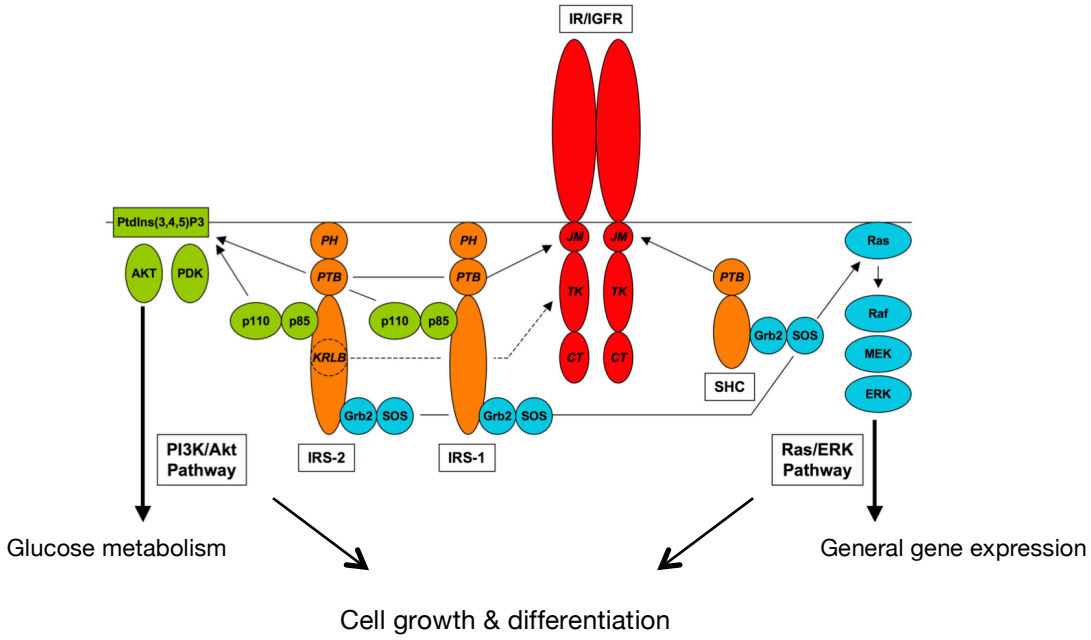


Figure 1. **Simplified scheme of canonical insulin/IGF signalling.**

Insulin activates the insulin receptor (IR) by inducing autophosphorylation on several tyrosine residues via the intrinsic tyrosine kinases (TK) located on the receptors β subunits. The activated receptor recruits insulin receptor substrate (IRS) and/or Src homology domain-containing (SHC) proteins. These bind to asparagine-proline glutamic acid phosphotyrosine (NPEY) in the β-subunit of the IR/IGFR via phophotyrosine-binding (PTB) domains. The binding of IRS proteins to activated receptors also depends on substrate binding to membrane lipids via pleckstrin homology (PH) domain. Recruitment of substrates results in their own phosphorylation on tyrosines, which in turn are docking sites for proteins with Src homology 2 (SH2) such as p85 and growth factor receptor-bound protein 2 (Grb2). Grb2 and p85 link the activated receptor to signalling modules like Ras/MAPK and PI3K/PKB, which in turn regulate gene expression, mitogenesis or glucose metabolism. Both pathways regulate cellular growth and differentiation. For a detailed description of adapter protein-dependent activation/regulation of PI3K/PKB and Ras/MAPK see text section 1.3.3. Image and figure legend adapted from [245]

1.3.2 The role of insulin signalling in β-cells

Since a long time, a possible autocrine effect of insulin on β-cells is a matter of controversial debate. Pancreatic β-cells express the IR [90, 106, 136, 276, 278] and stimulation of β-cells with insulin activates the insulin signalling cascade [161]. A number of studies indicate that insulin signalling indeed plays a critical role in various cellular processes in β-cells such as survival and growth [95, 146, 161, 285] Mice deficient for *Irs2* show β-cell loss already at birth and are not able to compensate for insulin resistance via increased β-cell mass [145, 288, 289]. It was therefore assumed, that the IRS2-PI3K/PKB signalling pathway must be essential for the regulation of β-cell mass, growth and survival.

Mice with a specific deficiency of insulin receptors in β-cells (βIRKO) have increased fasting insulin levels, are moderately glucose intolerant, and show impaired first-phase insulin secretion but have normal islet mass [147]. Mice specific deficient for IGF receptors in β-cells (βIGF1R) have a similar phenotype [148]. If both genes are deleted in β-cells (βDKO), mice show a more severe phenotype with loss of β-cell function and increased random fed blood glucose levels. β-Cell mass is decreased and the number of apoptotic cells is increased [272]. However, for generating these β-cell-specific knockout (KO) mice, the *rat insulin promoter* (*RIP*) was used to drive Cre-expression. This promoter is known to have a significant Cre-expression in the brain, including hypothalamus. It is well known that the central nervous system (CNS) has a high impact on metabolic homeostasis. Hence non-autonomous effects cannot be excluded [247, 286]. Additionally, there is no evidence for a positive effect on pro-insulin production and no evidence for autocrine regulation of insulin secretion in β-cells [222, 271]. Together these findings suggest that other ligands in addition to insulin/IGF can regulate β-cell proliferation and survival via IRS2 und downstream PI3K-PKB signalling to control the regulation of β-cell mass [222].

A possible candidate is the epidermal growth factor receptor (EGFR) signalling pathway. Deficiency in the EGFR leads to markedly reduced β-cell mass and severe diabetes in mouse models. Reduced activation of the signalling pathways of PKB and MAPK were detected in these models [103, 188, 189]. Additionally, mice with a deficiency in EGFR were not able to expand β-cell mass via proliferation during pregnancy or after high-fat diet (HFD) [153]. It can be assumed that the EGFR pathway is essential for β-cell proliferation in the insulin resistant state [103, 117, 163, 188, 189, 199, 263]. Additionally, mitogenic effect of GLP1 was absent after stimulation of defective EGFR [144]. GLP1 mediates β-cell survival and proliferation not only via PI3K-PKB pathway but also via activation of adenylyl cyclase, which in turn phosphorylates and activates CREB and that activates IRS2. Activation of IRS2 mediates β-cell proliferation and survival [125]. Glucose and other growth factors can activate IRS2 as well [12], whereas cytokines such as interleukin 1β (IL1β) and tumor necrosis factor α (TNFα) inhibit IRS2 via serine phosphorylation [220].

1.3.3 Insulin Receptor Substrate (IRS) adapter proteins

These evolutionary conserved proteins do not contain catalytic activity but act as adapters between activated membrane receptors and intracellular signalling pathways. They are crucial for many biological processes relating to proliferation, cellular growth, survival, cell differentiation and regulation of fuel metabolism [28, 93, 196, 255]. Six isoforms of IRS are known in rodents (IRS1-IRS6) [44, 154, 155, 256, 257]. The human genome does not encode IRS3. IRS proteins share a common structure: An NH_2-terminal pleckstrin homology (PH) domain that interacts with phospholipids or acidic motifs of proteins located in the plasma membrane and a phosphotyrosine-binding (PTB) domain that binds to asparagine-proline glutamic acid phosphotyrosine (NPEY) as present in the β-subunit of the IR. IRS proteins contain many tyrosines that when phosphorylated provide dockings sites for proteins with Src homology 2 (SH2) domains, such as p85, tyrosine protein phosphatase (SHPTP2) and Grb2 [283]. However, phosphorylation can also occur at serine/threonine sites, which in turn has inhibitory effects on IRS molecules. For example, stimulation with TNFα phosphorylates IRS1 at Ser307, inhibiting insulin-mediated tyrosine phosphorylation and causing IRS ubiquitination and finally its breakdown [134, 226]. Downstream components of insulin signalling like PKB, MAPK, glycogen synthase kinase 3β (GSK3β), mammalian target of rapamycin complex 1 (mTORC1), and ribosomal protein S6 kinase (S6K) are known inhibitors of IRS via feed-back phosphorylation on serine residues [26, 35, 313]. The function of IRS proteins in metabolic control has been studied in various mouse models. IRS1 and IRS2 are both widely expressed and mediate both, metabolic and mitogenic actions [243, 284]. IRS1 is the best-characterised IRS protein, contains 18 potential tyrosine phosphorylation docking sites for proteins containing SH2 domains and is widely expressed [256, 283]. Mice with a KO for *Irs1* show defects in embryonic and postnatal growth but are fertile and viable. They show resistance against insulin and IGF, but they avoid hyperglycaemia via compensatory increase of β-cell mass associated with hyperinsulinaemia [13, 259]. Additionally, *Irs1* deficient islets show dysregulated insulin production and secretion [149]. All this together indicates that IRS1 is involved in the regulation of metabolic and mitogenic functions in peripheral tissue [121, 260]. In islets, the function of IRS1 is confined to insulin secretion. It seems that IRS1 is not required in compensatory expansion of β-cell mass in the face of insulin resistance [196].

IRS2 shows 43% sequence identity relative to IRS1, but it contains 100 amino acids more than IRS1 and at least 20 confirmed tyrosine phosphorylation motifs. Like IRS1 it is widely expressed in various tissues. The region containing the PTB and PH domains is around 65% identical compared to IRS1 [232]. *Irs2* KO mice are insulin resistant and glucose intolerant starting from birth, but they show a constant increase of fasting blood glucose levels during their lifespan as a consequence of decreased β-cell mass [289]. However, isolated skeletal muscle from *Irs2* KO mice, showed normal insulin stimulated glucose uptake. Importantly, and in contrast to *Irs1* KO mice with impaired insulin sensitivity in skeletal muscle and compensatory increase in β-cell mass, *Irs2* deficient mice suffer from hepatic insulin resistance and β-cell failure. These observations indicate that IRS2 plays a pivotal role in liver insulin action and in insulin production in pancreatic islets, whereas IRS1 might be solely required for insulin action in skeletal muscle [13, 40, 112, 259, 295].

IRS3 was first found in adipocytes in rats. It is shorter than IRS1 and IRS2 and the PH and PTB domains

are around 50% identical to IRS1 and IRS2, respectively [294]. Mice with a deficiency in *Irs3* show normal glucose transport, glucose homeostasis and growth in adipocytes. [312]. However, overexpression of IRS3 in isolated adipocytes leads to enhanced recruitment of GLUT4 to the plasma membrane [130, 312].

IRS4 has 12 potential tyrosine phosphorylation residues and undergoes fast phosphorylation after insulin stimulation. It shows high expression in brain, thymus and pituitary. IRS4 plays no role in glucose metabolism and growth. Mice deficient for *Irs4* have minimally reduced weight, minimally reduced blood glucose levels, normal insulin plasma levels and show impaired glucose tolerance, but normal insulin tolerance [79]. Analysis of IRS5 and IRS6 show more structural identity (74%) to each other than to other IRS proteins in PTB domain. In mice, IRS5 is mainly expressed in lung, heart and kidney, whereas IRS6 is highly expressed in brain [44]. Up to date, no metabolically relevant functions have been described for IRS5 and IRS6.

It seems that only IRS2 plays an important role in the regulation of proliferation, survival and glucose-induced insulin secretion in β-cells from rat and human islets. Overexpression of IRS2 but not of IRS1 increases proliferation in isolated rat islets and protects isolated human islets from glucotoxicity. Additionally, only IRS2 increases glucose-stimulated insulin secretion after overexpression (GSIS) [190]. β-cell-specific up-regulation of IRS2 protects from the onset of diabetes due to a decreased rate of apoptosis and a compensatory increase of β-cell mass [109].

1.3.4 The PI3K/PKB signalling pathway

1.3.4.1 PKB regulates various cellular processes

PKB is part of the AGC family of protein kinases, with other members being proteinkinase A, G and C. Members of the AGC family have evolutionary conserved catalytic domains and show similarities in the mechanism of regulation [209]. Functions of the PI3K/PKB axis are highly context-specific and might depend on recruitment of different isoforms of PI3K (p85α, p110α-δ, PI3K-C2α-γ, vps34) and PKB (PKBα/AKT1, PKBβ/AKT2, and PKBγ/AKT3), respectively [178, 236, 237, 277]. PI3K-dependent conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) in the plasma membrane recruits PKB and results in its phosphorylation at Thr308 trough the upstream kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1) [83]. Phosphorylation at this site only partially activates PKB. For full kinase activation three other kinases can phosphorylate PKB at Ser473. DNA-dependent protein kinase (DNA-PK) phosphorylates PKB triggered trough DNA damages and the predominant kinase mTORC2 phosphorylates at Ser473 after stimulation with growth factors like insulin or IGF1. A further possible kinase is the ataxia telangiectasia mutated kinase (ATM) [33, 81, 236]. After its activation, PKB moves away from the plasma membrane and translocates to other cell compartments like the mitochondria, cytoplasm, or the nucleus where it regulates various downstream targets via phosphorylation. PKB promotes different cellular processes via regulation of various intracellular targets (shown in Figure 2). Inhibiting of AS160 (Rab-GTPase activating protein) allows GLUT4 translocation. PKB has an anti-apoptotic effect via inhibition of the Bcl-2-associated death

promoter (BAD), and mediates cellular growth via activation of mTORC [83, 236]. Additionally, PKB activates S6K and eIF4E-binding protein 1 (4EBP1), which in turn stimulates protein synthesis. PKB-dependent phosphorylation/inhibition of GSK3β promotes among numerous other effects glycogen deposition [222].

Various phosphatases can inhibit insulin-dependent activation of the PI3K-PKB axis (shown in Figure 2). Phosphatases (protein tyrosine nonreceptor type 1 (PTPN1, PTP1B)), lipid phosphatase and tensin homologue (PTEN), and protein phosphatase 2a (PP2A) inhibit IR, IRS1/2, PIP3 or PKB via dephosphorylation. Thioesterase superfamily members 4 (THEM4, CTMP) are binding partners of PKB that can also have inhibitory effects. Their functions in the regulation of glucose homeostasis remain unknown [68, 175, 236]. Additionally, insulin signalling can be downregulated by negative feed-back loops. Targets, such as GSK3β and mTORC1 inhibit IRS proteins via phosphorylation at serine residues, which in turn lead to ubiquitination and finally to its proteolytic breakdown [236].

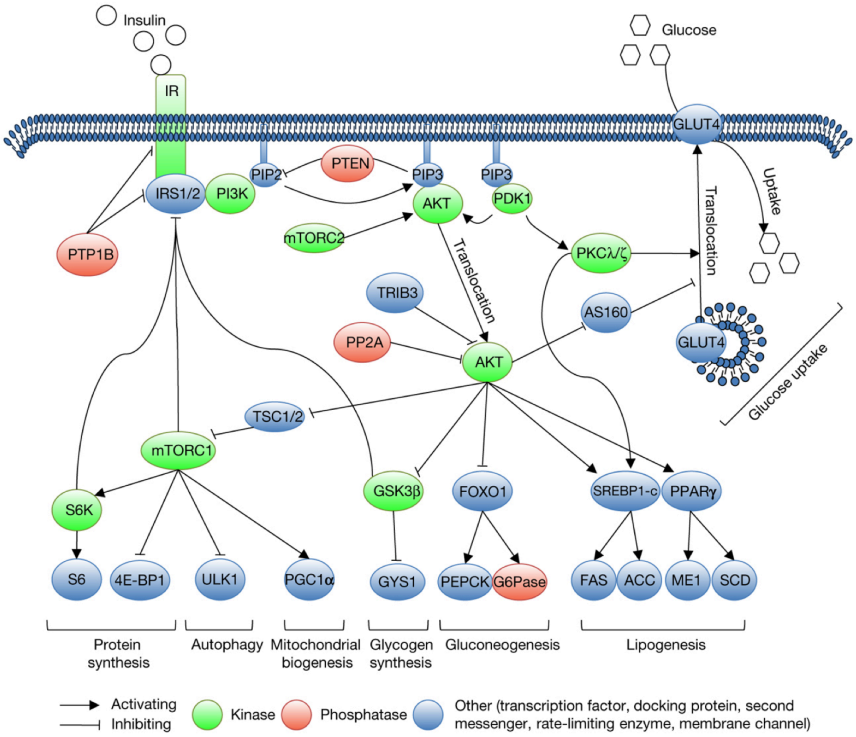


Figure 2. **PKB is required for many insulin-dependent functions.**

PKB mediates insulin action via inhibition or activation of various downstream targets. Inhibition of AS160 (AKT substrate 160) allow GLUT4 translocation and increases glucose transport. Inhibition of glycogen synthase kinase 3β (GSK3β) promotes glycogen synthesis. Downregulation of forkhead-O transcription factor 1 (FOXO1) stimulates activation of phosphoenolpyruvate carboxykinase 1 (PEPCK1) and the glucose-6-phosphatase (G6Pase) and promotes therefore gluconeogenesis. Up-regulation of lipogenic genes like peroxisome proliferator-activated receptor γ (*Pparγ*) and sterol regulatory element binding transcription factor 1 (*Srebp1c*) increases lipogenesis. Additionally, PKB activates mTORC1-dependent signalling via inhibition of tuberous sclerosis complex 1/2 (TSC1/2). This promotes protein synthesis via regulation of ribosomal protein S6 kinase (S6K) and eIF4E-binding protein 1 (4EBP1). mTORC1-dependent inhibition of unc-51-like kinase 1 (ULK1) blocks autophagy and activation of peroxisome proliferator-activated receptor γ coactivator α (PGC1α) induces mitochondrial biogenesis.
For detailed description of direct PKB inhibition or inhibition via IRS1/2 see text. Image and adapted figure legend are from [236] .

1.3.4.2 PKB isoforms have specific but also redundant functions in rodents

PKB isoforms show up to 80% identity in their amino acid sequence but are encoded by distinct genes that are located on different chromosomes. They are highly conserved in their catalytic PH domain and in the C-terminal regulatory subunit [105]. PKB isoforms vary in their expression pattern and their function, despite similar substrate specificity and similar protein structure. PKBα and PKBβ show high expression in most tissues, whereas PKBγ is mainly found in brain and testes [105]. All three isoforms are expressed in islets from humans and rodents [115, 193, 236]. The physiological functions and the tissue-specific effects of PKB isoforms have been intensively studied *in vivo* in mouse models with isoform-specific deficiencies or constitutive activation of individual isoforms. A selection of published mouse models and their phenotypes is shown in Table 1.

PKB isoform		Tissue	Phenotype	Ref.
Constitutive activation	α	Heart	Hyperplasia and hypertrophy of cardiomyocytes, improved myocardial contractility, enhanced glucose uptake	[52, 53, 142, 179, 180, 244]
		Prostate	Prostatic intraepithelial neoplasia (PIN) which further leads to bladder obstruction	[176]
		Thymus	Hyperplasia of thymocytes and development of lymphoma	[177]
		T-cells	Decreased apoptosis, hypertrophy, lymphoma, improved T-cell response and autoimmunity	[128, 195, 206, 219]
	β		Lymphoma	[185]
	α	Mammary gland	Erb2-mediated mammary tumour formation is accelerated, metastatic progression is suppressed, enhanced fat content in milk	[7, 118, 119, 240, 241]
		Pancreatic β-cells	β-cell hyperplasia and hypertrophy, improved glucose tolerance	[25, 270]
dominant-negative (kinase-dead)	all	Pancreatic β-cells	Impaired insulin secretion, decreased glucose tolerance, hyperglycaemia	[23]
Deletion	α	Whole body	Reduced body weight, increased perinatal mortality, reduced placenta development, protected from HFD-induced obesity and insulin resistance, Improved glucose tolerance and insulin sensitivity	[43, 47, 49, 297, 298]
		Brain	Not protected from HFD-induced obesity (only in whole body knock out mice)	[280]
		Skeletal muscle	No protection from HFD-induced obesity (only in whole body knock out mice)	[280]
	β	Whole body	Glucose intolerance, insulin resistance, hyperglycaemia, age-dependent loss of adipose tissue, most strains show increased β-cell mass, protected from HFD-induced hepatic steatosis.	[43, 48, 89, 108, 158]
		Hepatocytes	Protected from HFD-induced hepatic steatosis	[158]
	γ	Whole body	No metabolic phenotype, impaired brain development	[43, 75, 269]
	αβ	Whole body	High perinatal mortality, defects in growth, skin, bone and muscle development, impaired adipogenesis	[207]
	αγ	Whole body	High embryonic lethality, defects in growth, impaired development of nervous and cardiovascular systems	[297]
	βγ	Whole body	Impaired brain and testes development, decreased growth, reduced glucose and insulin tolerance	[71]

Table 1. **Phenotypes of mice carrying mutations in PKB isoform.**

Mice deficient for *Pkbα* show impaired placental development and weigh 15-30% less at birth than their wild type (wt) littermates. Additionally, they show higher perinatal mortality. *Pkbα* deficient mice are more insulin sensitive and glucose tolerant, show increased plasma glucagon levels and are protected from HFD-induced obesity. Ectopic expression of myristylated PKBα in β-cells increases islet mass via β-cell hypertrophy and hyperplasia. These mice show improved glucose tolerance and are protected from streptozotocin-induced diabetes [25, 43, 47, 49, 75, 269, 270, 280, 298].

Pkbβ deficient mice are glucose intolerant and insulin resistant with fasting hyperglycaemia and increased plasma insulin levels. Additionally, they undergo age-dependent reduction of adipose tissue [43]. Dependent on age, gender and strain, this metabolic impairment can culminate in β-cell failure and later in diabetes [89] but in most cases overt diabetes does not manifest due to sufficient compensatory expansion of β-cell mass and function [43, 48, 89].

Pkbγ deficient mice display defects in foetal growth and brain development. However, glucose

homeostasis is not affected in this model [43, 75, 269]. All these observations together indicate that only PKBα and PKBβ play a role in the regulation of glucose homeostasis, whereas PKBγ has no effect. Furthermore, the differences in phenotypes of mice deficient for individual isoforms of PKB point to non-redundant functions of PKB isoforms *in vivo*. In addition, studies in other tissues showed isoform-specific functions of PKB. In embryonic fibroblasts from mice only PKBα and not PKBβ promotes proliferation and cell migration [306, 311]. In skeletal muscle, siRNA-mediated silencing of PKBβ decreased glucose uptake and glycogen synthesis, whereas downregulation of PKBα had no effect. Furthermore, only silencing of PKBβ reduced lipid oxidation, while PKBα had an opposite effect [32].
There are also studies indicating redundancy: In adipocytes, gene silencing via siRNA of both, PKBα and PKBβ resulted in impaired insulin-stimulated glucose transport [126]. Indeed, the mild metabolic phenotype of *Pkb* deficient mouse models could also in part be due to redundant function of isoforms. In conclusion, depending on the respective context PKB isoforms can have specific and/or redundant functions [236, 237].

1.4 T2D is the most common diabetes form

Diabetes mellitus includes a group of metabolic diseases defined by chronic high blood glucose levels resulting from insufficient insulin production and/or impaired insulin action. Untreated chronic hyperglycaemia has severe long-term complications such as retinopathy, nephropathy, neuropathy and cardio- and cerebrovascular complications [31]. The American Diabetes Association (ADA) classifies diabetes in type 1 and type 2, other specific types of diabetes, and gestational diabetes. The highest prevalence with 90% of all diabetes forms has T2D. This diabetes form is characterised by chronic hyperglycaemia (fasting plasma glucose concentration ≥ 7mmol/L) due to a combination of insulin resistance and insufficient compensatory insulin secretion [3]. Genetic alterations as well as environmental factors are causes for T2D. Over 50 genes associated with T2D have been identified, but how they influence the pathogenesis of T2D remains still unclear. Most of them can be linked to β-cell function [131]. Risk factors for T2D are obesity (BMI ≥ 30), reduced physical activity, over-nutrition, urbanisation and aging. In early stages of the pathogenesis of T2D, dietary changes or increased exercise can avoid the progression of the disease. Chronic hyperglycaemia must be treated with oral antidiabetics (e.g. metformin or incretin analogues) or by insulin substitution [131]. In 2011, 366 million adults suffered from T2D and it is estimated that the number will increase to 7.7% of adult population in 2030 (552 million people) [172]. T2D is currently the 8th leading cause of death [4, 80]. The epidemic spread of T2D will be an enormous economic burden across the globe in the future.
5% of all diabetes patients are suffering from the insulin-dependent form of diabetes (T1D) [3]. The cause of this diabetes form is the autoimmune destruction of the pancreatic islets and β-cells, mediated by T-cells. Antibodies against insulin, glutamic acid decarboxylases, and protein tyrosine phosphatase trigger inflammation in the endocrine cells of the pancreas [59]. T1D is associated with inappropriate expression of genes regulating β-cell inflammation and apoptosis in pancreatic islets [231]. Humans with mutations in the histocompatibility leukocyte antigen (HLA) show higher risk for developing T1D. Additionally beside genetic predisposition, environmental triggers (viral pathogens, prenatal exposition to organochlorine

pollutants or vitamin D deficits) are postulated to stimulate the immune system to trigger islet destruction [55, 82, 253, 262]. Patients with T1D have to compensate for β-cell destruction for their entire life with exogenous insulin supplementation.

1.4.1 Causes and underlying mechanisms of insulin resistance

Under conditions of insulin resistance, insulin-induced glucose uptake in adipose and muscle tissue is reduced, gluconeogenesis in liver is enhanced whereas glycogen storage is suppressed. [234]. Insulin resistance is the primary cause of T2D and it occurs often long before T2D is diagnosed. Obesity and advanced age are major risk factors of insulin resistance [302].

Insulin resistance and T2D are associated with low-grade chronic inflammation, which includes macrophage infiltration and increased cytokine expression in adipose tissue, muscle and liver [104, 137]. In the obese state, adipocytes display morphological and functional changes. Their size is increased and they secrete an imbalanced mix of cytokines and adipokines [266]. Pro-inflammatory cytokines, such as TNFα, IL6 and leptin are increased whereas adiponectin is decreased. Leptin regulates satiety and energy intake. Obese subjects are often leptin resistant, as indicated by unsuppressed appetite despite high plasma leptin levels [120, 152]. Adiponectin has anti-inflammatory effects and in addition positively affects lipid and glucose metabolism, improves insulin sensitivity, and regulates appetite and body weight [166]. Obesity is also associated with macrophage infiltration and ER stress in adipose tissue with concomitant death of adipocytes (tissue inflammation). Chronically elevated glucose concentration, hypoxia and FFAs can induce ER stress and lead to activation of the unfolded protein response (UPR) and c-Jun N-terminal kinase 1 (JNK), a direct inhibitor of IRS proteins and known inducer of insulin resistance [204, 235]. Chronic low-level inflammation is believed to inhibit insulin signalling in adipocytes, myocytes and in hepatocytes [284, 299, 300, 303]. Cytokines, FFAs, reactive oxygen species (ROS) and hypoxia activate so-called stress kinases like e.g. IκBα kinase β (IKKβ) and JNK1 [114, 208, 301, 305]. These stress kinases can phosphorylate IRS proteins on Ser/Thr residues thereby inhibiting IRS-dependent signal transmission [8, 87, 208, 305, 309]. The principle mechanism underlying insulin resistance caused by chronic inflammation is exemplified by the effect of TNFα: TNFα binds to its p55 receptor and mediates phosphorylation of IRS at Ser307 [208]. This inhibits insulin-mediated tyrosine phosphorylation and causes IRS ubiquitination and finally its breakdown [134, 226]. Down regulation of IRS leads to decreased insulin-induced activity of PI3K/PKB and deterioration of insulin sensitivity. It can also lead to sustained activity of MAPK in liver and heart. Prolonged activation of MAPKs (ERK1/2, p38 and JNK) mediates mitogenesis and overgrowth, which in turn results in progression of obesity and also to cardiovascular dysfunction [64, 102, 218]. Mice with disrupted *Erk1* (*Mapk3*) fed a HFD show decreased adipocyte size and showed better insulin tolerance [30]. Down regulation of PKB increases the activity of forkhead-O transcriptions factor 1 (FOXO1) causing chronic hyperglycaemia and insulin resistance in liver [170, 310]. Furthermore, TNFα can also reduce the activity of peroxisome proliferator-activated receptor γ (PPARγ) and thus leads to reduced lipid synthesis and decreased storage of TGs [300, 303]. Thirdly, TNFα directly enhances lipolysis, which in turn leads to increased FFAs in plasma and decreased storage of TGs in adipocytes and liver [299].

Many other states like dysfunction of mitochondria, oxidative stress, hypoxia (affects cytokine expression), pregnancy, dyslipidaemia, fatty liver, genetic background and hyperinsulinaemia are also associated with insulin resistance [302]. During persistent insulin resistance, mitochondria are over activated because of increased energy disposal caused by glucose and FFAs overload. High levels of ATP inactivate AMP (adenosine monophosphate)-activated protein kinase (AMPK), which in turn leads to decreased glucose uptake. AMPK might also inhibit mTORC1-S6K induced inhibitory phosphorylation of IRS proteins and thus inactivation of AMPK could also reduce insulin sensitivity [236]. Enhanced levels of FFAs and glucose increase ATP production associated with the production of ROS [78, 84, 97, 164, 169, 223]. Saturated FFAs (linoleic acids, palmitate) can cause inflammation trough activation of IKK/NFκB pathway dependent on Toll-like receptor 4 (TLR4), a receptor for endotoxin (LPS) in adipocytes and macrophages. Furthermore, FFAs can be converted to diacyl glycerol (DAG) and ceramide. DAG is known to activate PKC isoforms. PKC activate JNK and IKKs known inhibitors of IRS1 activation [18, 38, 88, 159, 281]. Hyperinsulinaemia also leads to progressive insulin resistance especially in presence of FFAs. The insulin signalling pathway is controlled trough a negative feedback loop to avoid activation of insulin-induced stress responses. Insulin itself activates this pathway, which is responsible for the down regulation of IRS1 and IRS2 [54, 242, 299].

1.4.2 The role of β-cells in T2D and insulin resistance

1.4.2.1 Compensatory increase of β-cell mass can prevent the onset of T2D

Pancreatic β-cell compensation via expansion of β-cell mass and function can maintain normoglycaemia despite persistent insulin resistance [46, 124, 167, 249]. Obese patients display around 50% increase of β-cell mass compared to lean and healthy individuals [42]. Nutrients like glucose or FFAs, incretins (GLP1), insulin and other growth factors are known to stimulate β-cell growth and proliferation [66, 248, 249, 275]. There is strong evidence for a role of growth factor signalling pathways in compensatory islet response. As mentioned above, the IRS2-PI3K/PKB axis is believed to be an important contributor. PKB represses FOXO1 in β-cells leading to enhanced expression of pancreatic and duodenal homeobox 1 (*Pdx1*). *Pdx1* mediates β-cell proliferation and survival and increases insulin transcription [74, 124, 182, 200, 248]. In addition, PKB is a known inhibitor of proapoptotic proteins like BAD and thus activation of PKB can protect β-cells from apoptosis [124]. GLP1 can stimulate β-cell proliferation and survival via PKA/CREB-dependent activation of IRS2. Additionally, GLP1 activates PKB and PKCζ via transactivation of EGFR [41]. FFAs might stimulate β-cell proliferation via G-protein coupled receptor 40 (GPR40) that is highly expressed in β-cells [41, 122, 125].
Also β-cell function can be increased to compensate higher demand for insulin production. Four underlying mechanisms have been proposed: enhancement of β-cell glucose metabolism, FFA signalling, increased sensitivity for incretins and enhanced parasympathetic interventions in β-cells. Rats with leptin receptor defects (Zucker fatty rats) [314] show strong weight gain via enlarged and increased number of adipocytes, are insulin resistant but not hyperglycaemic. They showed up to 2 fold increase of total glucose utilisation via enhanced glucose oxidation as well as increased glucose flux via pyruvate

carboxylase (PC) [167]. FFAs increase β-cell function via FFAR1/GPR40-dependent increase in intracellular Ca^{2+} concentration, which in turn facilitates insulin release [122, 254]. How GLP1 increases β-cell function is not yet fully elucidated, but it is known that GLP1 supports FFA-dependent increase of GSIS in Zucker fatty rats [197]. Furthermore, GLP1 and glucose enhance transcription of the insulin gene via increasing cAMP, which in turn leads to increased transcription of the insulin gene mediated by CREB activation. High glucose concentrations increase also insulin levels via up-regulation of *Pdx1* [182, 183].

1.4.2.2 Mechanisms of β-cell failure

Pancreatic β-cell mass is often reduced in later stages of the pathogenesis of T2D. Post-mortem analysis of pancreata showed a decrease of 63% in β-cell mass in obese subjects with T2D compared to weight-matched non-diabetic controls [58]. However, detailed analysis of pancreatic sections showed no changes in rate of proliferation but increased rate of β-cell apoptosis in these subjects [42]. Increased glucose metabolism and increased levels of FFAs enhance oxidation reactions in mitochondria thus resulting in augmented levels of ROS [39, 69, 168]. Detoxification of ROS requires ATP and reduced levels of ATP decrease insulin secretion in β-cells. Furthermore, ROS increases β-cells apoptosis if not adequately detoxified [214, 224].

Chronically elevated circulating glucose increases insulin production to levels exceeding the capacity of the ER and can thereby induce ER stress in β-cells [133, 212]. That ER stress in β-cells can lead to β-cell failure is manifest in the Akita mouse model. Mice with a mutation in the *Insulin 2* gene (*Ins2*) show severe insulin deficiency and β-cell failure due to ER stress caused by misfolding of Proinsulin 2 [123]. In addition, hyperglycaemia can induce IL1β production/Fas-receptor expression in β-cells leading to β-cell death [62, 174]. Furthermore, impaired detoxification of lipids leads to accumulation of complex lipids and thus has cytotoxic effects on β-cells and increases apoptosis [215, 216].

Insulin is secreted together with islet amyloid polypeptide (IAPP). In patients with T2D, this polypeptide aggregates to intracellular amyloid micro fibrils, which in turn are believed to have toxic effects on β-cells, although the underlying mechanism remains unclear [198].

1.5 Aim of my thesis

Pancreatic β-cell mass adapts to the demand for insulin production and this can prevent the onset of T2D. IRS2 is indispensable for the maintenance but also for the compensatory expansion of functional β-cell mass. Downstream of IRS2 acts PKB, a global regulator of proliferation, apoptosis and cellular growth and is implicated to play a major role in the regulation of β-cell mass. Three isoforms of PKB exist: PKBα/AKT1, PKBβ/AKT2 and PKBγ/AKT3. While loss of individual isoforms leads only to mild islet phenotypes, overexpression of PKBα can increase β-cell proliferation in mice.

The aim of my thesis was to clarify the roles of PKB isoforms in the regulation of pancreatic β-cell mass and function.

My project consisted of three parts:

In a first part I studied if the functions of PKBα and PKBβ are evolutionary conserved in rodents and humans. To this end PKBα or PKBβ were overexpressed in isolated human islets. This part of the project is described in section 2.1 (adapted from manuscript submitted to *Archives of Physiology and Biochemistry*).

In a second part of my thesis I tested in mice with specific deletion of *Pkbα* in β-cells if *Pkbα* is required for the compensatory expansion of functional pancreatic β-cell mass under insulin resistance. This part is presented in section 2.2.

In a third part, I studied if PKB isoforms are differentially regulated by growth factors in β-cells. To this end I assessed the activation of PKB isoforms at Ser473 and Thr308 after stimulation with insulin, IGF or a combination of both. This last part is described in section 2.3.

2. Results

2.1 Specific and redundant roles of PKBα/AKT1 and PKBβ/AKT2 in human pancreatic islets

Maren G. Dietrich[1,2], Richard A. Zuellig[1], Brian A. Hemmings[3], Giatgen A. Spinas[1,2], Roger Lehmann[1], Oliver Tschopp[1,2], and Markus Niessen[1,2]

[1] Division of Endocrinology, Diabetes and Clinical Nutrition, University Hospital of Zurich, Switzerland

[2] Competence Center for Systems Physiology and Metabolic Diseases, Zurich, Switzerland

[3] Friedrich Miescher Institute (FMI) for Biomedical Research, Basel, Switzerland

Address correspondence and reprint requests to: Markus Niessen, PhD, Division of Endocrinology, Diabetes and Clinical Nutrition, Raemistrasse 100, 8091 Zurich Fax: +41-44-255'97'41; E-mail: markus.niessen@bluewin.ch

Abstract

Protein kinase Bα (PKBα)/AKT1 and PKBβ/AKT2 are required for normal peripheral insulin action but their role in pancreatic β-cells remains enigmatic as indicated by the relatively mild islet phenotype of mice with deficiency for either one of these two isoforms. In this study we have analysed proliferation, apoptosis, β-cell size and glucose-stimulated insulin secretion in human islets overexpressing either PKBα or PKBβ. Our results reveal redundant and specific functions. Overexpression of either isoform resulted in increased β-cell size but none of them affected insulin production and secretion. Proliferation and apoptosis of β-cells were only significantly stimulated and inhibited, respectively, by PKBα/AKT1. Importantly, overexpression of PKBα/AKT1 in dissociated islets increased the ratio of β-cells to non-β-cells. These results confirm our previous findings with rodent islets and strongly indicate that PKBα/AKT1 can regulate functional β-cell mass also in human islets.

2.1.1 Introduction

Mammals can adapt their capacity for insulin production to changes in demand [217]. Type 2 diabetes (T2D) manifests as this ability is lost [42]. How much insulin can be produced depends on total mass of β-cells located within the pancreatic islets of Langerhans and on how well they function (functional β-cell mass) [217]. Pancreatic β-cells compensate for increased demand via proliferation, cellular growth and reduced cell death [45]. However, the capacity for compensation is often limited by chronically elevated concentrations of D-glucose, high concentrations of saturated free fatty acids (FFAs), and low-grade inflammation that can induce apoptosis in β-cells. As a consequence, β-cell mass progressively decreases in later stages of the disease [131].

The proper regulation of maintenance and expansion of functional islet mass depends on insulin receptor substrate (IRS) 2 [196]. IRS proteins are components of canonical insulin/insulin-like growth factor (IGF) signalling and IRS1 and IRS2 are required for metabolic insulin action in muscle, liver, and adipose tissue, via activation of phosphoinositide 3-kinase (PI3K). It was shown that IRS2, but not IRS1, is required in β cells for proper regulation of survival, proliferation and function of islets [145, 289]. Indeed, IRS2 deficient mice are unable to compensate for peripheral insulin resistance and develop severe diabetes [145]. Furthermore, overexpression of IRS2 in islets can autonomously increase proliferation in β-cells and reduces high-glucose-induced β-cell apoptosis [109, 190]. These observations indicate that IRS2 is necessary and sufficient for the maintenance and compensatory increase of pancreatic β-cell mass.

Protein kinase B (PKB)/AKT is required for peripheral insulin action and functions as a global regulator of metabolism, differentiation, growth, proliferation, and survival in mammals. PKB is evolutionary highly conserved and an important component of IRS/PI3K signalling [236, 237]. Three isoforms of PKB exist in mammals; PKBα/AKT1, PKBβ/AKT2 and PKBγ/AKT3. The isoforms are encoded by separate genes, but share 80-85% sequence identity and the same structural organisation. They show distinct but overlapping expression patterns and differ in their function, despite their structural similarities [236]. Functions of PKB isoforms have been studied in mice deficient for individual isoforms [43, 48, 49, 75, 89, 107, 157, 269, 297, 298]. Mice with whole body knockout (KO) for *Pkbα* or *Pkbβ* show complementary respective phenotypes as for the regulation of glucose homeostasis: *Pkbβ*$^{-/-}$ mice are glucose intolerant and insulin resistant while *Pkbα*$^{-/-}$ mice show improved glucose tolerance and are more insulin sensitive. *Pkbγ*$^{-/-}$ mice show impaired brain development but normal metabolic control.

Although all three isoforms are expressed in human pancreatic β-cells [43], it is not clear if the regulation of functional β-cell mass is isoform-specific. Transgenic mice expressing constitutively active PKBα showed a two-fold increase in β-cell proliferation [25] and overexpression of PKBα in rat insulinoma cells (INS1) protects from FFA-induced apoptosis [291]. Furthermore, insulin secretion was found to be impaired after downregulation of PKB activity in pancreatic β-cells, suggesting PKB is required for normal β-cell function [23]. When overexpressed in isolated islets *in vitro*, only overexpression of PKBα but not PKBβ increased β-cell proliferation [43]. In contrast to gain of function (GOF), *Pkb* deficient mice show no or only mild islet phenotypes. Whole body deficiency for *Pkbβ* even results in β-cell compensation and hyperinsulinaemia. The activation/phosphorylation of PKB isoforms was studied in β-cells overexpressing IRS2 [43]. Only PKBα but not β or γ was activated by IRS2 and IRS2-dependent enhancement of β-cell

proliferation was impaired in islets lacking *Pkbα* but normal in the case of deficiency for *Pkbβ* or *γ* indicating that PKBα is regulated and required downstream of IRS2 in islets.

The aim of this present study was to clarify in human islets if GOF for the metabolically relevant isoforms (PKBα and PKBβ) exerts differential effects in β-cell function, proliferation, apoptosis, and size, respectively. To induce GOF, PKBα and PKBβ were overexpressed from adenoviral vectors in intact human islets cultured on extra-cellular matrix-coated dishes. In order to facilitate the identification of β-cells, islets were also dispersed prior to transfection and analysis. Function was studied in intact islets by assessing glucose-stimulated insulin secretion (GSIS) and insulin production. In both intact and dispersed islets only PKBα increased significantly β-cell proliferation, respectively. Neither ectopic expression of PKBα nor of PKBβ affected insulin production or secretion. However, both isoforms increased β-cell size, but only PKBα protected β-cells from apoptosis and increased the percentage of β-cells to non-β-cells.

2.1.2 Materials and methods

2.1.2.1 Human islets

Human islets were obtained from the Juvenile Diabetes Research Fund (JDRF) and the European Consortium for Islet Transplantation's (ECIT) "Islets for Research Distribution Program".
Islets were cultured in CMRL medium (Invitrogen, Carlsbad, USA) containing 5 mM D-glucose (Invitrogen, Carlsbad, USA), 10% FCS (HyClone Laboratories Inc., Logan, USA), 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen, Carlsbad, USA).
Around 50 islets were plated on dishes coated with extracelllular matrix (ECM) derived from bovine corneal cells (Novamed, Jerusalem, Israel) and allowed to attach and flatten out for three days prior to the start of experiments [132]. Each condition was carried out in triplicate.
β-cells cannot be unambiguously distinguished from non-β-cells in intact human islets cultured on ECM-coated dishes. To this end islets were dispersed by collagenase digestion as described by Linetsky et al. [165]. Around 40’000 cells were plated on ECM-coated dishes in triplicate and allowed to attach for 24 hours.

2.1.2.2 Rat islets

Islets from Sprague-Dawley rats (200-250g) were isolated according to Gotoh et al. [96]. In brief, the pancreatic duct was perfused with and digested at 37°C in 10 mL cold Hank’s Balanced Salt Solution (HBSS) (Sigma-Aldrich, Saint Louis, MO) containing collagenase (10 units) (VitaCyte, Indiana, USA), rat protease (1 unit) (Serva GmbH, Heidelberg, Germany), 1.5 mg DNAse (Boehringer Mannheim, Mannheim, Germany), and 0.02 M HEPES (Invitrogen, Carlsbad, USA). Islets were purifed from digested tissue via a density gradient (Histopaque-1119; Sigma-Aldrich, Saint Louis, MO) and subsequent hand picking. Isolated islets were cultured on ECM-coated dishes (Novamed, Jerusalem, Israel) in RPMI 1640 medium containing 11.1 mM D-glucose (Invitrogen, Carlsbad, USA), 10% FCS (HyClone Laboratories Inc., Logan, USA), 100 units/mL penicillin, 100 units/mL streptomycin, and 40 g/mL gentamicin (Invitrogen, Carlsbad, USA). Islets were cultured from three days prior to transfection with adenoviral constructs.

2.1.2.3 Adenoviral transfection

Transfections and adenoviral constructs were described previously [43]. In brief, adenoviral constructs for expression of Hemmagglutinin (HA)-tagged PKBα, PKBβ, or GFP were supplied by Vector BioLabs (Philadelphia, USA). Intact or dispersed islets on ECM plates were exposed to viral particles at multiplicities of infection (MOI) of 500 to 1000 or 100 to 200, respectively, for two days. Afterwards islets or cells were washed twice with PBS to remove residual viral particals.

2.1.2.4 Western blotting

Cells were lysed in a buffer containing 50 mM HEPES, 140 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 3 µg/mL aprotinin, 3 µg/mL 10 mM sodium fluoride, 1 mM disodium

pyrophosphate, and 1 mM sodium orthovanadate. Protein concentration was determined using the Bicinchoninic Acid Assay (BCA Protein Assay Kit, Thermo Scientific, Rockford, USA). SDS-PAGE and Western blotting was performed with the NuPAGE system from Invitrogen (Carlsbad, USA) according to the manufacturers instructions. Equal amounts of protein were loaded on NuPAGE Novex 4 to 12% gradient Bis-Tris Gels and afterwards transferred onto nitrocellulose mebranes (GE Healthcare, Buckinghamshire, England). The membranes were incubated with 1° antibodies overnight at 4 °C and incubated with 2° antibody for 2h at RT. Lumi-Light Western blotting substrate (Roche, Basel, Switzerland) was used for visualisation of signals in a Fuji LAS-3000 ChemImager (Fujifilm, Tokio, Japan). Bands were quantified using AIDA software (Raytest, Germany). Antibody against HA was derived from a hybridoma cell line (12CA5). An antibody against PKBβ was purchased from Cell Signalling Technology (Beverly, MA, cat no. 2964). PKBα was detected using an antibody from BD Bioscience (Franklin Lakes, USA cat no. 610860). Phosphorylated-PKB was detected using antibodies against p-Ser473 and p-Thr308 (both from Cell Signalling Technology, Beverly, USA cat no. 9271 and 5106, respectively). An antibody against actin was purchased from MilliporeChemicon International (Billerica, USA). Secondary HRP-linked antibodies were used (anti-mouse from Santa Cruz Biotechnology, Santa Cruz, USA cat no. 2005, and anti-rabbit from Bio-Rad, Hercules, USA, cat no. 172-1019).

2.1.2.5 Glucose-stimulated insulin secretion and determination of total insulin content

Around 50 islets per dish in triplicate were incubated in Krebs-Ringer bicarbonate (KRB) buffer with 0.5% BSA and 2.8 mM D-glucose for 1 h at 37°C, stimulated with 16.7 mM D-glucose for 1 h at 37°C and thereafter incubated for 1h at 37°C at 2.8 mM D-glucose. This last step allows assessment of how fast islets can re-adjust to lower concentration of D-glucose after stimulation with high concentration of D-glucose. To determine total insulin content insulin was extracted from islets by incubating overnight in acid-alcohol (0.18M HCl in 70% ethanol). Insulin in supernatants and acid-alcohol extracts was measured by radioimmunoassay (RIA, CIS Bio international, Gif-Sur-Yvette, France) and normalised to protein content as measured by the Pierce Protein BCA assay kit (Thermo Scientific, Rockford, USA). For rat islets, identical procedure was performed as described for human islets. Insulin concentration was measured by ELISA (Rat Insulin ELISA, Mercodia, Uppsala, Sweden).

2.1.2.6 Analysis of apoptosis and proliferation

Apoptosis was assessed using the In Situ cell death detection kit from Roche (Basel, Switzerland). Intact or dispersed islets were cultured for four days in the presence or absence of 2ng/mL interleukin 1β (IL1β) (Prospec, Ness-Ziona, Israel, cat no. cyt-394). Cells were fixed in 4% formalin for 30 minutes. Islets or β-cells were visualised by co-staining for insulin (mouse anti-insulin; Sigma-Aldrich, Saint Louis, USA, cat no. I2018, clone K36AC10). To assess proliferation, the BrdU Labeling and Detection Kit II (Roche, Basel, Switzerland) was used. Intact or dispersed islets on ECM plates were cultured for 4 days in the presence of 5-Bromo-2'-deoxyuridine (BrdU) (10 μM) and were fixed in 4% formalin for 30 minutes at room temperature. Co-staining for insulin (mouse anti-insulin; Sigma-Aldrich, Saint Louis, USA, cat. I2018,

clone K36AC10) and DAPI allowed detecting of islets or β-cells. Intact islets: The number of islets as well as mean islet- and β-cell size was determined and used to calculate the total number of islet cells per dish. Apoptotic/proliferating islet cells were counted, expressed in percent of total number of cells and normalised to GFP control. For assessment of β-cell proliferation and apoptosis in dispersed islets at least 2000 cells were analysed.

2.1.2.7 Assessment of β-cell size, ratio of β-cells to non-β-cells

β-cell size was determined mophometrically in dispersed islets by co-staining for insulin and DAPI by measuring the area of 60 cells. The ratio of β-cells to non-β-cells was determined by counting insulin-positive and non-insulin-positive cells at five random positions on the plate (at least 600 cells were counted).

2.1.2.8 Microscopic analysis

An Axioplan 2 from Zeiss with Axio Vision software (Zeiss, Göttingen, Germany) were used for microscopic analysis. β-cell size and ratio of β-cells to non-β-cells was assessed using Image J software (National Institutes of Health, Bethesda, Maryland). Microscopy pictures in this publication were obtained on an epifluorescence microscop (Eclipse Ti, Nikon insturments with μManger, [76]).

2.1.2.9 Statistical analysis.

Data are shown as mean ± standard error of mean (SEM) unless indicated otherwise. For comparison of two groups, unpaired Student's *t-tests* were performed. Analysis of variance (ANOVA) with Bonferroni's post hoc test was used for multiple-comparisons. Results were considered to be statistically significant when P value was ≤ 0.05 *; $P \leq 0.001$ **; $P \leq 0.001$ ***.

2.1.3 Results

2.1.3.1 Overexpression of PKBα, PKBβ or GFP in human islets

Intact islets and dispersed islet cells from seven human donors were used in this study. Age, gender, body mass index (BMI), cold ischemia time, and cause of death are listed for each donor in Table 1. In all except two cases cause of death was cerebral bleeding. The mean donor age was 50.7 (ranging from 21-61) and mean BMI [kg/m^2], was 24.07 ± 3.0. Mean purity of the used isolations was 76% (at least 60%) and islets of all preparations were glucose responsive. Cold ischemia time (time of suppressed blood supply until start of isolation) was in a similar range for each donor. Only preparations that showed such high purity and were glucose-responsive were used in this study.

A reliable transfection scheme was established for every batch of viral preparations obtained, to ensure expression of transgenes to similar levels. Representative Western blots and immunostainings are shown in Figure 1. Similar expression levels of PKBα and PKBβ in intact islets and also after dispersion of islets were achieved (Figure 1A). Figure 1B visualises ectopic expression relative to endogenous PKBα and PKBβ. Importantly, overexpression of PKB isoforms resulted in increased levels of p-PKB(Ser473) and p-PKB(Thr308) (Figure 1C). Fluorescent-microscopic analysis of *AdV-GFP* transfected islets revealed that transfection efficiency was typically around 80% in dissociated islets and 60% in intact islets (Figure 1D).

2.1.3.2 Overexpression of PKBα or PKBβ does not affect β-cell function

β-cell function was assessed with intact islets cultured on ECM plates, by measuring accumulated insulin in supernatants of islets shifted from low to high to low glucose concentrations and in acid/alcohol extracts of lysed islets.

Without transfection insulin secretion was increased 4.0 ± 2.4 fold (n=7, Table 1 and Figure 2A) in high glucose concentration. Transfection with *AdV-GFP*, *AdV-PKBα* or *AdV-PKBβ* did neither change insulin secretion nor insulin content (Figure 2A and B, respectively).

In rat islets, GSIS was affected neither after *AdV-PKBα* nor after *AdV-PKBβ* transfection. *AdV-PKBβ* transfection showed only a tendency toward increase of total insulin content (Supplementary figure 1).

2.1.3.3 Overexpression of PKBα increases proliferation but PKBα and PKBβ can increase β-cell size

In intact islets (Table 2) overall basal proliferation was 0.2% ± 0.13% (n=5). Proliferation was unchanged after transfection with *AdV-GFP*, indicating that transfection per se did not significantly influence proliferation (Figure 3A). Islets also did not show any change in proliferation after transfection with *AdV-PKBβ* but proliferation was increased 3.64 ± 0.55 fold ($P \leq 0.001$) in islets transfected with *AdV-PKBα*. In dispersed islets basal rates of proliferation for β-cells and non-β-cells of were 0.16% ± 0.11% and 0.7% ± 0.7%, respectively (Table 2). β-cell proliferation varied significantly between donors (range between 0.018% ± 0.018% and 0.27% ± 0.064%). Proliferation of non-β-cells and of β-cells remained unchanged after transfection with *AdV-GFP* (Figure 3B). There was no significant change in proliferation of non-β-

cells after transfection with *AdV-PKBα* or *AdV-PKBβ* (not shown). β-cell proliferation was significantly increased 3.53 ± 0.4 fold ($P \leq 0.01$) only after transfection with *AdV-PKBα*. Transfection with *AdV-PKBβ* merely resulted in a trend towards increased proliferation of β-cells (2.17 ± 0.59 fold, n.s). β-Cell size was determined microscopically with dispersed islets (Table 2). Mean β-cell size was 119.65 μm^2 ± 31.3 μm^2 (n=6) with significant variation between donors (range between 89.9 μm^2 ± 5.7 μm^2 and 159.3 μm^2 ± 8.24 μm^2). Overexpression (Figure 4) of GFP did not result in any change whereas overexpression of PKBα or PKBβ increased β-cell size by around 50% (1.55 ± 0.12 fold and 1.45 ± 0.05 fold, respectively; $P \leq 0.001$; $P \leq 0.01$, respectively).

2.1.3.4 Overexpression of PKBα protects from apoptosis and increases the ratio of β-cells to non-β-cells

Basal and IL1β-induced apoptosis were assessed in intact and dispersed islets by staining for TUNEL (Table 2 and Figure 5). In intact islets (Table 2) the rate of apoptosis was 0.14% ± 0.09% (n=5). Transfection with *AdV-GFP* did not increase apoptosis significantly relative to non-transfected controls (Figure 5A). Without IL1β transfection with *AdV-PKBα* or *AdV-PKBβ* did not change apoptosis compared to GFP control. Culturing the islets in the presence of IL1β increased apoptosis significantly 2.41 ± 0.2 fold in untransfected islets and 1.5 ± 0.13 fold ($P \leq 0.01$) in islets transfected with *AdV-GFP*. Only transfection with *AdV-PKBα* resulted in inhibition of IL1β-induced apoptosis (Figure 5A).
After dispersion, basal apoptosis was 0.36% ± 0.32% (n=4) for non-β-cells and 0.9% ± 0.62% (n=4) for β-cells (Table 2). IL1β did not increase apoptosis significantly in dispersed islets (not shown). Transfection with *AdV-GFP* increased apoptosis of non-β-cells and β-cells (2.56 ± 0.64 and 1.55 ± 0.25 fold, respectively) relative to non-transfected controls (Figure 5C and E). Transfection with *AdV-PKBβ* only reduced apoptosis of non-β-cells significantly (0.6 ± 0.054 fold). Transfection with *AdV-PKBα* reduced apoptosis of non-β-cells (0.4 ± 0.05 fold, $P \leq 0.001$) and β-cells (0.47 ± 0.05 fold, $P \leq 0.001$).
We determined the ratio of β-cells to non-β-cells after dispersion of islets and culturing them for 4 days by staining for DAPI and insulin to detect all nuclei and to identify β-cells, respectively (Figure 5F). The mean percentage of β-cells in dissociated islets was 46.14% ± 17.13% (n=6). Variations between donors were found (range between 18.3% ± 0.8% and 59.1% ± 1.1%) Transfection with *AdV-PKBα* increased the percentage of β-cells by 10.4% ± 2.4% *(*$P \leq 0.01$). Transfection with *AdV-GFP* or *AdV-PKBβ* had no effect. In rat islets, apoptosis was only assessed in intact islets. Only transfection with *AdV-PKBα* protected from IL1β-induced apoptosis (Supplementary figure 2).

Donor and islet isolation information								
Donor	1	2	3	4	5	6	7	Mean ± SD
Age [y]	55	58	59	41	61	60	21	51 ± 14.8
Gender	♀	♀	♂	♂	♂	♂	♀	n.a.
BMI [kg/m^2]	25.4	19.2	24.5	21.6	24.5	24.5	28.8	24 ± 3
Cause of death	cerebral bleeding	cerebral bleeding	cerebral bleeding	anoxia	cerebral bleeding	cerebral bleeding	anoxia	n.a.
Cold ischemia [h]	7	6	7.5	6	7.5	5	7	6.6 ± 0.9
Purity [%]	85	85	85	70	70	80	60	76 ± 9.9
Stimulation index	5.7 ± 1.21	8.4 ± 1.4	2.4 ± 0.07	2.04 ± 0.13	2.0 ± 0.14	5.0 ± 0.43	2.8 ± 0.6	4.0 ± 2.4
β-cells [%]			41.3 ± 4.1	59.1 ± 1.1	53.5 ± 2.3	58.5 ± 3.5	18.3 ± 0.8	46.1 ± 17
Islet size [µm^2]	46’922 ± 5600	24’220 ± 1944	46’081 ± 4698	54’731 ± 493	45’423 ± 623	33’286 ± 3029	64’351 ± 6492	45’002 ± 13’186

Table 1. **Donor information.**
Age [y], gender, BMI [kg/m^2], cause of death, cold ischemia time [h], and purity of the islets [%] are listed as transmitted by the islet isolation facility that provided the islets. Stimulation index (fold increase of insulin secretion at high glucose (16.7 mM) compared to low glucose (2.8 mM), relative numbers of β-cells per islet [%], and islet size (average area of islets [um^2]) were determined in the course of this study. n.d.: not determined, n.a.: not applicable. Where applicable a mean and SD/SEM were calculated.

Rate of proliferation, apoptosis and β-cell size of non-transfected islets			
Proliferation [%]	intact		0.21 ± 0.13
	dispersed	non-β-cells	0.7 ± 0.7
		β-cells	0.16 ± 0.11
β-cell size [µm²]	dispersed		119.6 ± 31.3
Apoptosis [%]	intact		0.14 ± 0.09
	dispersed	non-β-cells	0.36 ± 0.32
		β-cells	0.9 ± 0.62

Table 2. Rate of proliferation, apoptosis, and β-cell size for non-transfected islets.

Numbers of proliferating and dying cells were determined by BrdU-incorporation or TUNEL, respectively, for intact or dispersed islets cultured on ECM-coated dishes for 4 days. Data are shown as mean percentage ± SD (n ≥ 4). β-cell size was determined microscopically in dispersed islets by measuring the area of at least 60 cells on 3 different plates.
Data are shown as mean ± SD (n ≥ 4).

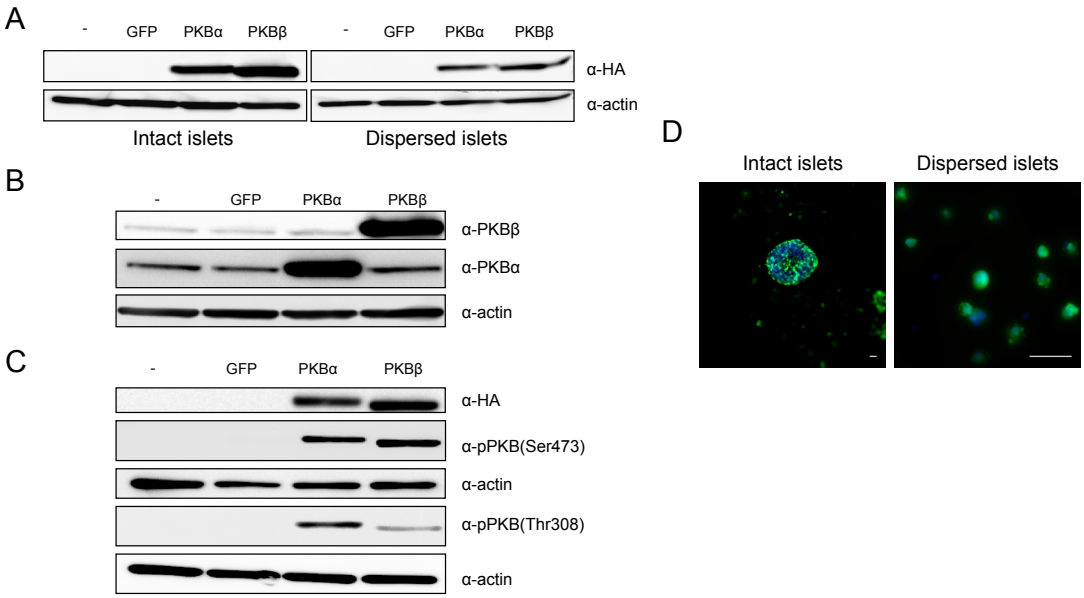


Figure 1. **Ectopic expression of PKBα, PKBβ or GFP in human islets.**

(A-C) Western analysis of lysates obtained from intact or dispersed islets cultured on ECM-coated dishes and transfected with adenoviral constructs to express HA-tagged fusion proteins. Membranes were incubated with (A) anti-HA antibody to verify that PKBα and PKBβ were expressed to similar levels and with (B) anti-PKBα or anti-PKBβ to visualise ectopic expression relative to endogenous expression and with (C) anti-phospho-PKB(Ser473) and phospho-PKB(Thr308) to assess activation. Actin was detected as a control. (D) Islets were dispersed and cultured on ECM-coated dishes. Fixed cells were stained with DAPI and inspected under a fluorescence microscope to count the number of cells expressing GFP. Scale bars, 50 μm.

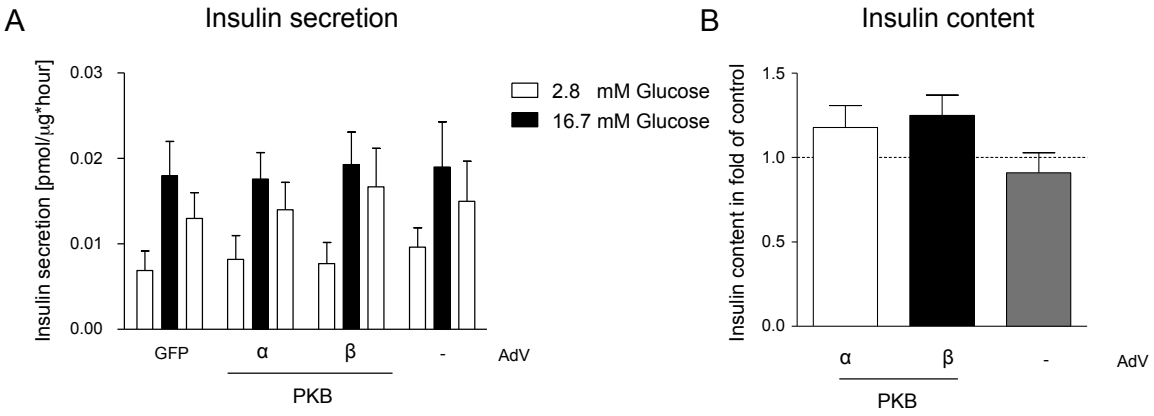


Figure 2. **Overexpression of PKBα or PKBβ does not affect β-cell function.**

Intact islets on ECM plates were transfected with *Adv-PKBα*, *Adv-PKBβ*, or *Adv-GFP*. (A) Accumulated insulin in supernatants of islets shifted from low to high to low glucose concentrations (GSIS) and (B) total insulin extracted from islets were determined by RIA. Data are shown as (A) mean or as (B) mean in fold of GFP control ± SEM (n=6).

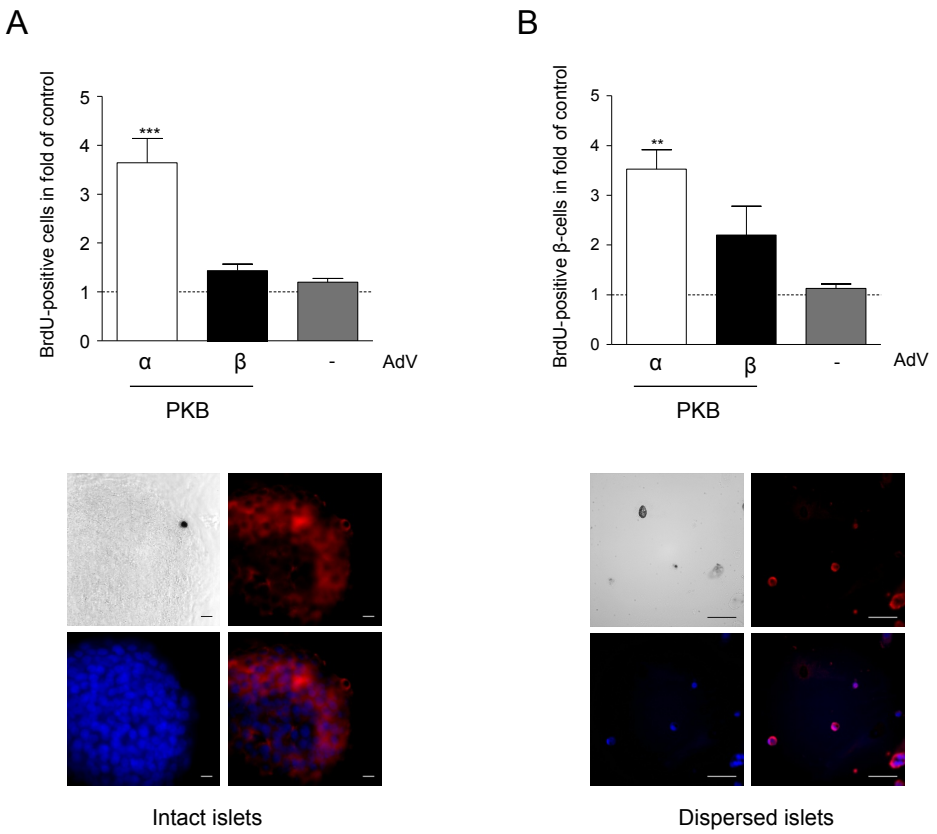


Figure 3. **Overexpression of PKBα increases proliferation in islets and in β-cells.**

Intact (A) or dispersed islets (B) on ECM-coated dishes were transfected with *Adv-PKBα*, *Adv-PKBβ*, or *Adv-GFP*. Proliferation was assessed by BrdU-incorporation over 4 days of culture. Representative microscopic images from intact and dispersed islets are shown below the bar graphs. BrdU-positive cells are stained brown, co-staining for insulin (red) allowed detection of islets or β-cells. DAPI was used to visualise nuclei (blue). Scale bars, 25 µm. Data are shown as mean in fold of GFP control ± SEM (n=4). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

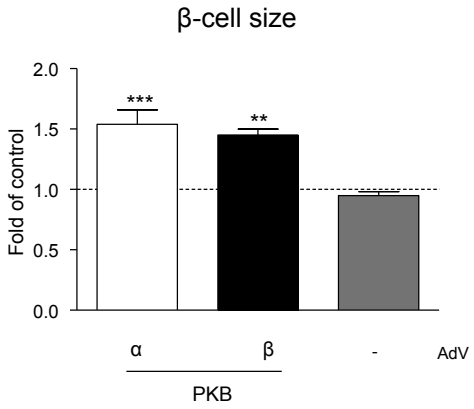
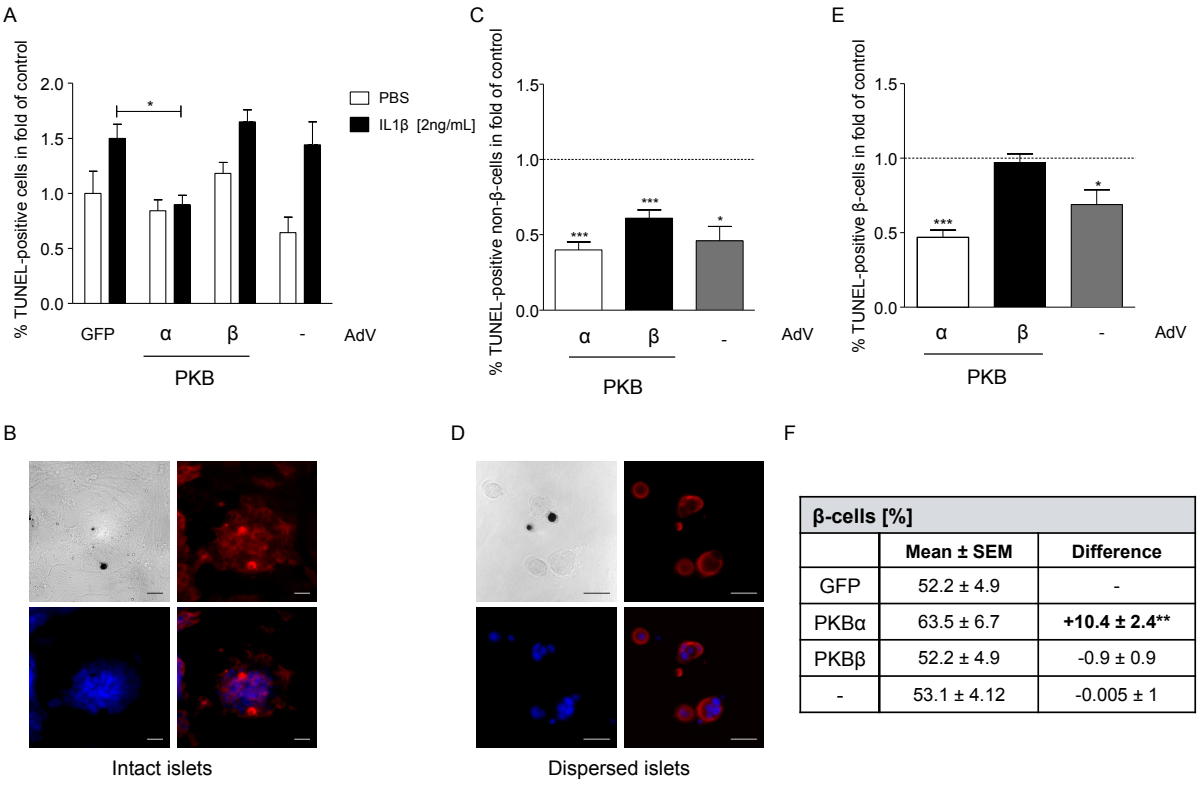


Figure 4. **Overexpression of PKBα or PKBβ increases β-cell size.**

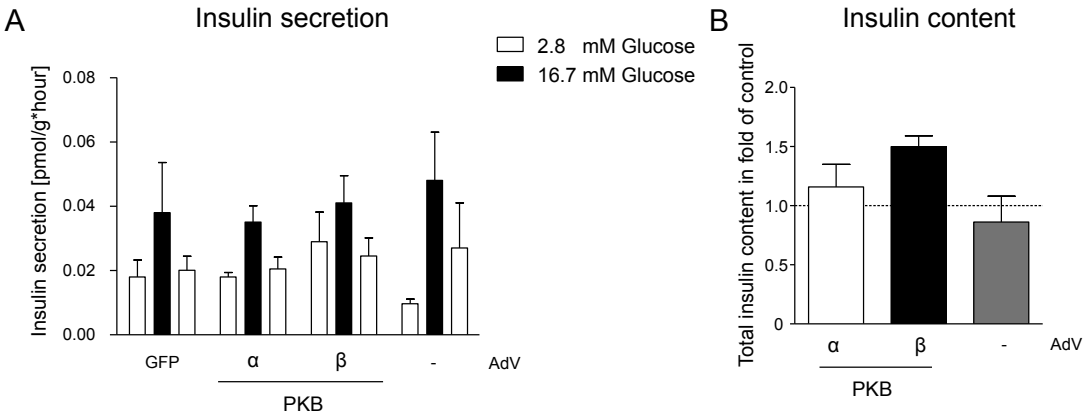
Dispersed islets on ECM-coated dishes were transfected with *Adv-PKBα*, *Adv-PKBβ*, or *Adv-GFP*, fixed and immunostained for insulin (red) and DAPI (blue). β-cell size was determined microscopically by measuring the area of at least 60 cells on 3 different plates per condition. Data are shown as mean in fold of GFP control ± SEM (n=4). **, $P \leq 0.01$; ***, $P \leq 0.001$.



β-cells [%]		
	Mean ± SEM	**Difference**
GFP	52.2 ± 4.9	-
PKBα	63.5 ± 6.7	**+10.4 ± 2.4****
PKBβ	52.2 ± 4.9	-0.9 ± 0.9
-	53.1 ± 4.12	-0.005 ± 1

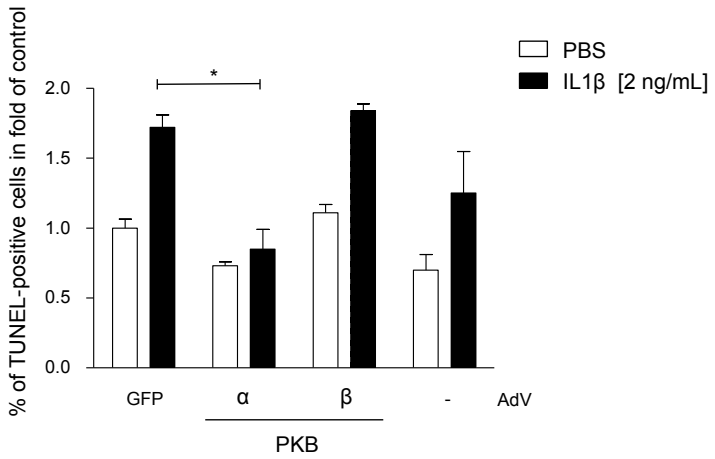
Figure 5. Overexpression of PKBα protects from apoptosis and increases the ratio of β-cells to non-β-cells.

Intact (A and B) or dispersed islets (C-F) on ECM-coated dishes were transfected with *Adv-PKBα*, *Adv-PKBβ*, or *Adv-GFP*. Apoptosis was assessed by TUNEL-staining after 4 days of culture in the presence or absence of IL1β (2ng/mL). Representative microscopic images from intact and dispersed islets are shown below the bar graphs. TUNEL-positive cells are stained brown, co-staining for insulin (red) allowed detection of islets or β-cells. DAPI was used to visualise nuclei (blue). Scale bars, 25 µm. Data are shown as mean in fold of GFP control ± SEM (n=4). (A) IL1β induced apoptosis only in intact islets. (F) Percent of β-cells relative to total number of counted cells presented as mean ± SEM (n=4). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



Supplementary figure 1. **β-cell function remains unaffected in rat islets overexpressing PKBα or PKBβ.**

Intact rat islets on ECM plates were transfected with *Adv-PKBα*, *Adv-PKBβ*, or *Adv-GFP*. (A) Accumulated insulin in supernatants of islets shifted from low to high to low glucose concentrations and (B) total insulin extracted from islets were determined by ELISA. Data are shown as (A) mean or as (B) mean in fold of GFP control ± SEM (n=3).



Supplementary figure 2. **Only overexpression of PKBα protects rat islets from IL1β-induced apoptosis.**

Intact rat islets on ECM-coated dishes were transfected with *Adv-PKBα*, *Adv-PKBβ*, or *Adv-GFP*. Apoptosis was assessed by TUNEL-staining after 4 days of culture in the presence or absence of IL1β (2ng/mL). TUNEL-positive cells are stained brown, co-staining for insulin (red) allowed detection of islets. DAPI was used to visualise nuclei (blue). Only overexpression of PKBα protects islets from IL1β-induced apoptosis. Data are shown as mean in fold of GFP control ± SEM (n=3). *, $P \leq 0.05$

2.1.4 Discussion

The molecular genetic analysis of PKBα and PKBβ in mice had revealed that these two structurally very similar isoforms exert opposing effects on peripheral insulin sensitivity: Deficiency for *Pkbα* results in improved but deficiency for *Pkbβ* in reduced insulin sensitivity [43, 280]. Furthermore, analysis of pancreatic islets from *Pkb* deficient mice *in vitro* and *in vivo* had suggested that the regulation of functional islet mass is mainly regulated by *Pkbα*. With this study we wanted to assess if PKBα or PKBβ can regulate functional β-cell mas also in human islets.

The activation of PKB isoforms is regulated via two phosphorylation sites, Ser473 and Thr308 dependent on mTORC2 and PI3K, respectively. Overexpression of PKBα or PKBβ resulted in increased intracellular levels of PKB phosphorylated at Ser473 as compared to *AdV-GFP*-transfected controls. This increased phosphorylation points to increased activation of PKB and is in line with the notion that overexpression does indeed result in GOF for PKB. Furthermore, our Western blot analysis suggests that PKB phosphorylation at Thr308 was more increased by overexpression of PKBα. If this was true, isoform-specific functions might be associated with differential phosphorylation of the two regulated phosphorylation sites also in human pancreatic islets, as was previously observed in skeletal muscle of mice [32]. Unfortunately, we were unable to perform a more thorough analysis of the phosphorylation of PKB at Thr308 as availability of islets limited our Western blot analysis.

Overexpression experiments as performed in this study are bound to technical limitations that are worth pointing out. For example the potency of isoforms can only be compared if similar ectopic expression levels are achieved. To this end we adjusted the respective MOIs in our experiments to obtain similar expression levels. Furthermore, ectopic expression was set to levels well below saturation where increase or decrease of the MOI would result in increase or decrease of ectopic expression, respectively. To account for possible adenoviral effects we analysed islets transfected at different MOIs and with different respective expression levels (not shown). Proliferation, apoptosis and function were clearly regulated dependent on the respective isoform that was overexpressed and not dependent on MOI or viral preparation.

Additionally, to use human islets is associated with further limitations. There is a high degree of variation due to donors with different age, BMI, sex, death causes, and health status at the time of death. Only limited numbers of donors are available. Indeed, rate of proliferation and apoptosis as well as β-cell size varied significantly between donors. Despite all of this, data reached high statistical significance in many of our experiments, which further indicates that there are indeed functional differences between PKB isoforms in pancreatic islets.

Our findings with human pancreatic islets are in good agreement with previously published observations with mouse islets: Both experimental settings indicate that GOF for PKBα, but not for PKBβ, is sufficient to increase functional islet mass. In human islets overexpression of PKBα increased β-cell proliferation and protected from cytokine-induced apoptosis more than overexpression of PKBβ, importantly only overexpression of PKBα significantly increased the ratio of β-cells to non-β-cells. Interestingly, IL1β did not induce apoptosis after dispersion of islets. IL1β is known to stimulate intra-islet immunoreaction mediated by macrophages and other islet-resident immune cells [61]. We can only assume, that after

collagenase digestion these cells were absent or spatially separated in our cultures, resulting in failure of IL1β to induce apoptosis. Alternatively, different concentrations of IL1β might be more effective in inducing β-cell apoptosis.

However, as islet mass is normal or even increased in *Pkbα* and *Pkbβ* deficient mouse models, both isoforms might only be required for β-cell compensation but dispensable for maintenance of islet mass [43, 196]. To determine if PKBα (and/or PKBβ) are required in human islets, loss of functions (LOF) experiments e.g. with siRNA will have to be performed. As human islets are difficult to obtain, such experiments to inhibit expression of PKB isoforms were beyond the scope of this study.

In contrast to β-cell apoptosis and proliferation, β-cell size was increased by PKBα and PKBβ to the same extend indicating that PKB isoforms can have specific and redundant functions within a single cell type. As the currently available antibodies to detect activation/phosphorylation of PKB are not isoform-specific it is not possible to correlate activation with function by Western blotting or immunostaining. This should also be taken into account in the analysis of PKB activation in peripheral tissues such as muscle and adipose tissue where PKBα and PKBβ might even perform opposite roles with respect to the regulation of insulin sensitivity. Future experiments should include a detailed analysis of the function of PKBα in the regulation of pancreatic β-cell mass in LOF models with specific deficiency for PKBα in β-cells. Such a model would allow to study the role of PKBα in functional β-cell mass in a metabolic context dependent of nutrition status, age and gender of mice.

2.2 The role of PKBα in the compensatory expansion of pancreatic β-cell mass under insulin resistance

M G. Dietrich [1,2]; R A. Zuellig[1]; B A. Hemmings[3]; G A. Spinas[1,2]; O. Tschopp[1,2] ; M. Niessen[1,2],

[1] Division of Endocrinology, Diabetes & Clinical Nutrition, University Hospital of Zurich, Switzerland

[2] Competence Center for Systems Physiology and Metabolic Diseases, Zurich, Switzerland

[3] Friedrich Miescher Institute (FMI) for Biomedical Research, Basel, Switzerland

Abstract

The protein kinase B (PKB)/AKT is indicated to play a major role in the regulation of pancreatic β-cell mass. Whereas loss of *Irs2* results in β-cell failure, loss of *Pkb* isoforms results merely in insulin secretion defects with very little β-cell loss. Based on previous studies it was suggested that IRS2 can specifically regulate PKBα, but not PKBβ or γ, to induce compensatory expansion of islet mass. According to this view, maintenance of islet mass does not require IRS2-dependent regulation of PKB. The goal of this study was to test this hypothesis. To this end a new mouse model was generated with specific deletion of *Pkbα* in β-cells (*βpkbaKO*). Mice were made insulin resistant by feeding a high-fat diet. Under normal chow diet *βpkbaKO* mice developed glucose intolerance only later in adult live at the age of 26 weeks. Under HFD *βpkbaKO* showed deteriorated glucose tolerance at earlier age in live. Plasma insulin levels from HFD-fed *βpkbaKO* mice were decreased compared to control littermates, indicating failure of β-cell compensation. First analysis of pancreas morphology revealed decreased β-cell area relative to total pancreas area in *βpkbaKO* mice compared to control littermates. Our data are in line with the hypothesis that PKBα plays a pivotal role in β-cell compensation under insulin resistance but is dispensable for maintenance of islet mass under normal conditions.

2.2.1 Introduction

Insulin resistance is believed to be the first step in the pathogenesis of type 2 diabetes (T2D) and in many cases it is diagnosed long before the manifestation of overt diabetes. Insulin resistance without diabetes can occur as the capacity of insulin production can increase by expansion of functional pancreatic β-cell mass. Such compensatory increase of islet mass ensues mainly via hypertrophy, enhanced proliferation and decreased cell death of β-cells [45]. Under conditions of overt T2D, β-cell mass is often decreased by about 60% [156]. An increased rate of apoptosis is thought to be the main cause of decreased β-cell mass. The causes for β-cell failure are complex and not fully understood yet, but glucotoxicity, lipotoxicity, proinflammatory cytokines, endoplasmatic reticulum (ER) and oxidative stress are known contributors [63], possibly via serine phosphorylation of the adapter protein insulin receptor substrate 2 (IRS2*)* [220]. Serine phosphorylation of IRS2 leads to its ubiquitination and proteolytic breakdown. IRS2 is pivotal for the proper regulation of functional β-cell mass [12, 190, 196] and its downregulation leads to inhibition of various downstream targets, responsible for β-cell survival [220]. Mice deficient for *Irs2* are insulin resistant and show severe β-cell failure followed by development of hyperglycaemia and finally diabetes [145, 289]. However, mice deficient for *Irs1* are insulin resistant but they are able to compensate the higher demand for insulin via compensatory increase of β-cell mass [13, 259]. This indicates, that IRS2 and not IRS1 is required for the maintenance and the compensatory increase of β-cell mass. In addition, only overexpression of IRS2 and not IRS1 in isolated rat islets leads to increased β-cell proliferation and protects from high glucose-induced β-cell death [190].

Protein kinase B (PKB/Akt) is a global regulator of growth, proliferation and apoptosis in various cell types and is implicated to play a major role also in the regulation of β-cell mass [45, 46, 124, 167, 249]. Insulin and other β-cell growth factors are known to be potent activators of PKB. The serine/threonine kinase PKB belongs to the AGC family including protein kinase A, C and G. Three isoforms of PKB are expressed in mammals: PKBα/AKT1, PKBβ/AKT2, and PKBγ/AKT3. PKB isoforms show similarities in their protein sequences (80-85%) and in their structural organisation but are encoded by distinct genes on different chromosomes [290]. They differ in their tissue-specific expression. High expression of PKBα is found in most tissues, PKBβ is prominent in insulin-targeted tissues, whereas PKBγ is highly expressed in brain and testes [89]. The relative importance of individual PKB isoforms for specific biological functions remains unclear, but evidence for non-redundant roles of PKB isoforms in insulin-target tissues is accumulating [237]. In skeletal muscle, only PKBα promotes lipid metabolism. In skeletal muscle and adipocytes, only PKBβ promotes glucose uptake and glucose metabolism [16, 32]. Metabolic roles of PKB isoforms were analysed in mice with isoform-specific deletion. *Pkbα*$^{-/-}$ -mice show lower blood glucose levels, improved glucose tolerance during glucose-and insulin tolerance tests, and significantly increased insulin-dependent glucose incorporation in adipocytes [43, 280]. Mice without *Pkbα* are protected against high-fat diet (HFD)-induced obesity and insulin resistance [280]. Analysis of pancreas morphology showed no change in β-cell but slightly increased α-cell area. *Pkbβ*$^{-/-}$-mice are glucose-and insulin intolerant and show age-related loss of adipose tissue, but analysis of pancreas showed a compensatory increase of islet mass and increased proliferation of β-cells [43, 48, 89]. *Pkbγ*$^{-/-}$-mice show no metabolic phenotype [43, 75, 269]. All these previous findings indicate that of the three PKB isoforms

only PKBα and PKBβ are required for glucose homeostasis. Analysis of islet morphology suggests, however, that PKB isoforms are largely dispensable in the regulation of maintenance of β-cell mass [43]. In contrast, IRS2 is required for maintenance and compensatory increase of β-cell mass, because mice deficient for *Irs2* are born with reduced β-cell mass and develop diabetes as they are insulin resistant in liver but fail to compensate the higher demand for insulin via increased β-cell mass [145]. Based on this striking discrepancy in severity of islet phenotype in *Irs2* versus *Pkb* deficient mice it was postulated that at least two separate downstream pathways must be controlled by IRS2 [196]: One for the regulation of maintenance and the second for the regulation of compensation. As maintenance of islet mass remains unaffected in *Pkb* deficient mice it was predicted that PKB is only necessary for the compensatory increase of islet mass downstream of IRS2.

Previous findings indicate that PKBα is the major isoform required downstream of IRS2 for β-cell compensation [43]. IRS2-induced proliferation was decreased in *Pkbα* deficient islets compared to wild type (wt) islets and *Pkbβ* deficient islets indicating that PKBα but not PKBβ is required downstream of IRS2. Furthermore, it was found that only PKBα was phosphorylated at Ser473 after overexpression of IRS2 in rat insulinoma cells (INS1).

The aim of this study was to test in mice if PKBα is required for the compensatory expansion of functional pancreatic β-cell mass under insulin resistance. To this end, a new β-cell-specific conditional knock out (KO) model was generated. To induce β-cell compensation mice were made insulin resistant by feeding a HFD. We assessed the metabolic phenotype of such mice under chow and HFD at different time points. Islet morphology was studied in pancreatic sections. Mice deficient for *Pkbα* in β-cells showed age- and obesity-dependent reduction of glucose tolerance caused due to insufficient compensation of functional β-cell mass.

2.2.2 Materials and Methods

2.2.2.1 Mice

The mouse strain with the *Pkba* gene flanked by *LoxP* sites (*Akt1*$^{flox/flox}$) (kindly provided by Zai Chang, Nanjing University, China) will be published elsewhere. To specifically delete *Pkba* in β-cells *Akt1*$^{flox/flox}$ mice were crossed to mice that carried a construct for expression of Cre under the control of the rat *Ins2* gene promoter (*RIP*): (B6*; D2-Tg(Ins2-cre)*^{1Herr}, termed *RIP-Cre* [111], kindly provided by Pedro Herrera, University of Geneva, Switzerland). *Akt1*$^{flox/flox}$ females were crossed to *RIP-Cre; Akt1*$^{flox/+}$ males to generate *RIP-Cre*; *Akt1*$^{flox/flox}$ (*βpkbaKO*) and *Akt1*$^{flox/flox}$ offspring (control), respectively. 6 weeks old mice *βpkbaKO* mice and control littermates were fed a standard chow diet (Provimi Kliba SA, Kaiseraugst, Switzerland) or a high-fat high-sucrose diet (HFD, Surwit Research Diets, New Brunswick, NJ, with 58% of calories from fat, 26% from carbohydrates, and 26% from protein; D12331) for 22 weeks. At the age of 28 weeks, mice were killed and pancreas, fat, muscle, liver and brain were dissected for further analysis. Both, females and males were analysed. Mice were housed in groups of up to 5 littermates with 12h dark-light cycle at 21°C and were allowed free access to food and water. Breeding of mice and all conducted procedures were carried out in agreement with Swiss Animal Protection laws and were approved by the appropriate authorities. All experiments with the exception of the analysis of pancreas morphology were performed for males and females. HFD feeding did not lead to significant weight gain in females. No significant differences between female *βpkbaKO* mice and controls were found (data not shown).

2.2.2.2 Determination of glucose and insulin in blood samples

D-glucose was measured in blood drawn from the tail vein of mice by using a glucose meter (Accu-chek Aviva, Roche, Switzerland). Blood samples for determination of insulin were collected from the tail vein or by cardiac puncture of euthanized animals, supplemented with aprotinin (Sigma-Aldrich, Saint Louis, Missouri, USA), centrifuged and plasma was immediately frozen. Insulin was measured with an ELISA Kit (Ultra Sensitive Mouse Insulin ELISA Kit, Crystal Chem, Downers Grove IL, USA).

2.2.2.3 Intraperitoneal glucose and insulin tolerance tests (ipGTT, ipITT)

For ipGTT, mice were fasted overnight (18h). 2g of glucose per kg bodyweight (20% solution, Bichsel, Switzerland) was injected into the peritoneum. For ipITT, mice were fasted for 3h and insulin was injected at 0.75 U/kg BW for females and 1U/kg BW for males (Actrapid, Novo Nordisk, Sweden). Mice at the age of 6 weeks, 12 weeks, 18 weeks or 26 weeks were tested. D-glucose levels were measured at time points 0, 15, 30, 45, 60, 90 and 120 min. For measuring insulin release during a glucose challenge, mice were fasted overnight and glucose was injected (2g/ kg bodyweight). Furthermore, area under the curve (AUC) was calculated to assess differences in ipGTT and ipITT.

2.2.2.4 Tissue dissections and assessment of fat depots

Sacrificed animals were weighed and blood was collected after cardiac puncture. Pancreas, liver, muscle, and fat were dissected, weighed and snap-frozen in liquid nitrogen or fixed in 4% formalin for 12h prior to embedding in paraffin. Fat from four different depots: epididymal-, peritoneal-, inguinal- and mesenteric fat was analysed.

2.2.2.5 Immunohistochemistry and morphometric analysis of pancreas

Pancreatic sections were deparaffinised, rehydrated, blocked and incubated overnight at 4°C with antibodies against insulin (mouse monoclonal, DAKO, Glostrup, Denmark) or glucagon (rabbit polyclonal, DAKO, Glostrup, Denmark). Secondary antibodies conjugated with FITC-or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) respectively, were used. Nuclei were visualised by DAPI staining (Sigma-Aldrich, Saint Louis, Missouri, USA).
Stained sections were analysed using a Zeiss Axioplan 2 microscope and Axio Vision software (Zeiss, Göttingen, Germany). Islet size and the number of islets per area of tissue, β- and α-cell area, and β- and α-cell size were determined.

2.2.2.6 Islet isolation and culture

Islets were isolated by collagenase (Worthington Biochemical Corporation, Lakewood, USA) digestion of pancreas from mice [227]. For further purification, islets were handpicked and dissociated into single cells by another collagenase digestion step. For culturing *in vitro*, dissociated islets were plated on extracellular matrix-coated (ECM)-dishes (Novamed, Jerusalem, Israel) and allowed to attach overnight. Islets or β-cells were cultured in RPMI 1640 medium containing 11.1 mmol/L D-glucose (Invitrogen, Carlsbad, California), 10% FCS (Hyclone Laboratories Inc., Logan, Utah, USA), 100 units/mL penicillin, 100 µg/mL streptomycin and 40 g/mL gentamycin (Invitrogen, Carlsbad, California, USA).

2.2.2.7 Western blotting

Tissues or cells were lysed in 50 mM HEPES, 140 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 3 µg/mL leupeptin, 3 µg/mL aprotinin, 10 mM sodium fluoride, 1 mM disodium pyrophosphate and 1 mM sodium orthovanadate. Protein concentration of lysates was determined by the Bicinchoninic Acid Assay (BCA Protein Assay Kit, Thermo Scientific, Rockford, USA). Western blotting was performed using the NuPAGE system from Invitrogen (Invitrogen, Carlsbad, California, USA) according to the manufacturer's recommendations. Equal amounts of protein were diluted in NuPAGE LDS Sample Buffer and Sample Reducing Agent and loaded onto NuPAGE Novex 4-12% Bis-Tris Gels. After electrophoresis was complete, separated protein bands were transferred onto nitrocellulose membranes (Amersham, GE Healthcare, Buckinghamshire, England). The membranes were incubated overnight at 4°C with primary antibody and afterwards for 2h with secondary antibody at room temperature.

Lumi-Light Western Blotting Substrate (Roche, Basel, Switzerland) was used to visualise signals. Signals were detected and quantified with a LAS-3000 ChemImager (Fujifilm, Tokyo, Japan) and AIDA software (Raytest, Germany). Antibodies against PKBβ, PKBγ and total-PKB were purchased from Cell Signalling Technology (Beverly, Massachusetts, USA). An antibody against PKBα was from BD Bioscience (Franklin Lakes, New Jersey, USA). As a loading control actin was visualised (Millipore-Chemicon International, Billerica, Massachusetts, USA). Secondary antibodies against mouse were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA) and against rabbit from BioRad (Hercules, California, USA).

2.2.2.8 Statistical analysis

Data are shown as mean ± SEM. To test significance, unpaired Student's *t*-tests (two-tailed) were performed. $P \leq 0.05$*; $P \leq 0.01$**; $P \leq 0.001$**.

2.2.3 Results

2.2.3.1 Validation of the new mouse model

A schematic overview of the strategy to generate our model is shown in Figure 1A. β-cell-specific deletion of *Pkbα* is induced by Cre expression under control of the *RIP* promoter. No change in body weight and random-fed plasma glucose were observed between $Pkb\alpha^{flox/flox}$ and wt mice at the age of 22 weeks (data not shown), indicating that the function of *Pkbα* is intact without Cre expression. Western blot analysis was performed to confirm that PKBα was normally expressed in $Pkb\alpha^{flox/flox}$ (controls) mice but absent or strongly reduced in β-cells of *βpkbαKO* mice. Liver from whole body knock out mice ($Pkb\alpha^{-/-}$) and from wt littermates ($Pkb\alpha^{+/+}$) was used as negative and positive control, respectively. Indeed, PKBα was normally expressed in all examined tissues of $Pkb\alpha^{flox/flox}$. In contrast PKBα was absent in lysates from β-cells of *βpkbαKO* but its expression was unchanged in liver, muscle, fat and brain. Expression of PKBβ and PKBγ was not affected (Figure 1B).

2.2.3.2 *βpkbαKO* mice show impaired glucose tolerance at 26 weeks of age

Body weight, blood glucose (random-fed and after fasting), ipGTT and ipITT were assessed at 6, 12, 18, and 26 weeks of age. Results are presented in figures grouped according to age (Supplementary figure 1-3; Figure 2). No differences in body weight, random fed and fasting (18h) blood glucose levels were found between *βpkbαKO* mice and controls (Supplementary figures 1A-3A; Figure 2A). In ipGTT, blood glucose levels were unchanged at 6, 12 and 18 weeks of age (Supplementary figures 1B-3B). However, 26 weeks old *βpkbαKO* mice showed significantly higher blood glucose levels with significantly increased AUC (+ 24.2% ± 5.7%, $P \leq 0.05$) during ipGTT (Figure 2B). IpITT revealed no changes in blood glucose levels between *βpkbαKO* mice and controls at all time points (Supplementary figures 1C-3C; Figure 2C). Furthermore, a tendency towards increased blood glucose in *βpkbαKO* mice after 3h of fasting at age 18 and 26 weeks was observed (Supplementary figures 3A; Figure 2A).

2.2.3.3 HFD accelerates the onset of impaired glucose tolerance in *βpkbαKO* mice

To induce insulin resistance, mice were fed a HFD starting from the age of 6 weeks up to the age of 28 weeks. Mice under HFD were analysed at the age of 12, 18 and 26 weeks corresponding to 6, 12 and 20 weeks of HFD, respectively. Data are presented as for animals under chow diet in Figures 3-5. Animals under high-fat diet had significantly increased body weight and reduced glucose tolerance compared to mice under chow diet (Figures 3BC-5BC). Random-fed and fasted blood glucose levels remained unchanged up to 20 weeks of HFD between controls and *βpkbaKO* mice (Figures 3A-5A). Glucose tolerance of *βpkbaKO* mice deteriorated significant compared to controls throughout the entire course of the HFD period (Figures 3D-5D). Already after 6 weeks of HFD glucose tolerance was significant reduced in *βpkbaKO* mice compared to controls (Figure. 3D). In ipITT, glucose levels were not changed after 6 weeks of high-fat diet (Figure 3E). After 12 weeks of HFD, significantly lower blood glucose levels were found in *βpkbaKO* mice compared to controls at 90 min and 120 min after insulin administration (Figure. 4E). Significantly lower AUC (-27.25% ± 5.17%, $P \leq 0.05$) was observed in *βpkbaKO* mice after 20 weeks of HFD (Figure 5E).

2.2.3.4 HFD-induced increase of plasma insulin is absent in *βpkbαKO* mice

Insulin levels were determined in chow- and HFD-fed mice at the age of 28 weeks (Figure 5F). No significant difference in plasma insulin was detected between *βpkbaKO* mice and controls under chow diet. Control mice fed a HFD showed significantly increased plasma insulin levels compared to chow-fed mice (7.54 ng/mL ± 2.5 ng/mL vs. 2.18 ng/mL ± 0.39 ng/mL, $P \leq 0.05$). *βpkbaKO* mice under HFD showed significantly lower plasma insulin levels (2.5 ng/mL ± 0.58 ng/mL, $P \leq 0.05$) compared to controls (7.54 ng/mL ± 2.5 ng/mL).

2.2.3.5 *βpkbαKO* mice are partially protected from HFD-induced weight gain

Weight gain under HFD was similar in *βpkbaKO* mice and controls up to 15 weeks of HFD (21 weeks of age). At later time points *βpkbaKO* mice were significantly lighter (Figure 6A). At the age of 28 weeks (22 weeks of HFD), mice were sacrificed and the weights of various fat depots, pancreas and liver were assessed. Under chow died no significant difference in total fat weight was observed between *βpkbaKO* mice and controls. Analysis of adipose tissue showed significantly increased total fat mass in percent of bodyweight after HFD in *βpkbaKO* mice (+6.05% ± 0.019%, $P \leq 0.001$) and controls (+9.4% ± 0.84%, $P \leq 0.001$). This significant increase affected all analysed fat depots (epididymal-, inguinal-, mesenteric-, and peritoneal fat).

However, *βpkbaKO* mice had significantly lower total fat weight in percent of bodyweight (12.5% ± 0.61%; $P \leq 0.05$) compared to controls (15.3% ± 0.84%) (Figure 6B). Peritoneal fat was significantly reduced in *βpkbaKO* mice (-0.46% ± 0.08%, $P \leq 0.05$) compared to controls while in mesenteric fat (-0.5% ± 0.124%), inguinal fat (-1.5% ± 0.42%) reduction did not reach significance (Figures 6D-6F). Epididymal fat depots were similar between both groups (Figure 6C).

Liver and pancreas weight in percent of bodyweight were similar between both groups under chow diet

and under HFD, respectively. Significant differences between liver and pancreas weight could be detected between chow- and HFD-fed mice. Pancreas and liver weight in percent of body weight is decreased in HFD-fed *βpkbαKO* mice (pancreas: -0.17% ± 0.088%; $P \leq 0.001$; liver: -1.13% ± 0.08%; $P \leq 0.001$) and controls (pancreas: -0.27% ± 0.039%; $P \leq 0.05$; liver: 0.6% ± 1.25%; $P \leq 0.05$) compared to aged matched mice under chow diet (Figures 6G-6H).

2.2.3.6 Morphometric analysis of pancreas showed decreased β-cell area in *βpkbαKO* mice

Islet morphology was assessed in 28 weeks old mice under chow diet. Pancreatic sections were stained for insulin, glucagon, and DAPI. Islet number per mm^2 of pancreas area was decreased (-40% ± 10.7%; n.s) in *βpkbαKO* mice (Figure 7A). In addition, β-cell and α-cell area were reduced in *βpkbαKO* mice (β-cell area: -46.1% ± 12.9%; n.s; α-cell are: -46.9% ± 13.8%; n.s) (Figures 7B-7C), whereas islet size proportion, β-cell and α-cell size remained unchanged (Figures 7D-7F). Preliminary analysis of pancreatic sections from HFD-fed animals indicates strongly reduced islet size and number in *βpkbαKO* compared to controls (not shown).

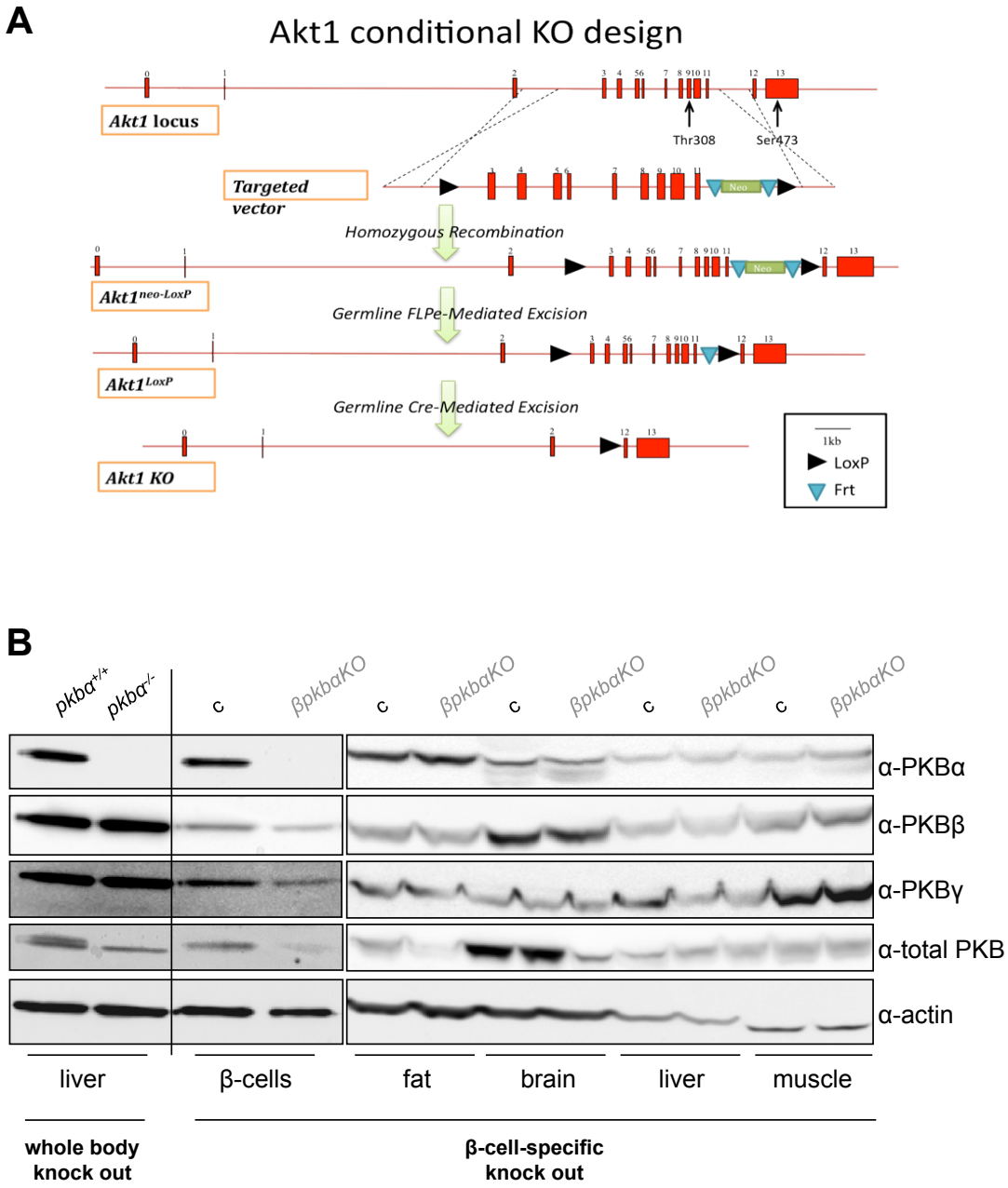


Figure 1. PKBα is lost exclusively in β-cells.

(A) Strategy for β-cell-specific deletion of *Pkba*: *LoxP* sites were inserted into the *Pkba* locus between exons 2/3 and 11/12. The catalytic domain including the regulated Thr308, are included between the *LoxP* sites. *LoxP* sites flanking exons 3-12 and containing a neomycin cassette were inserted by homologous recombination into the *Pkba* locus. The Neo cassette was removed by FLP-mediated excision resulting in a functional *Pkba* locus. Dependent on conditional Cre expression exons 3-11 can be deleted resulting in a null mutation in *Pkba*.

(B) Western blot analysis to assess expression of PKBα, PKBβ, PKBγ and total levels of PKB in β-cells, fat, brain, liver and muscle from *βpkbaKO* mice and controls. Lysates from liver of whole body knock out mice (*Pkba*$^{-/-}$) and wt littermates (*Pkba*$^{+/+}$) were used as negative and positive control, respectively. PKBα is missing in β-cells, while expression in fat, brain, liver, and muscle is similar in *βpkbaKO* mice and controls. Expression of PKBβ and PKBγ remained unchanged, whereas total PKB expression in β-cells was reduced, but normal in fat, brain, liver, and muscle in *βpkbaKO* mice compared to controls. Actin was detected as loading control.

A

	control	*βpkbαKO*
Weight [g]	32.8 ± 1.0	33.5 ± 0.6
Blood glucose [mmolL]		
random-fed	8.35 ± 0.24	8.64 ± 0.25
3h fasting	6.9 ± 0.4	7.4 ± 0.4
18h fasting	4.9 ± 0.3	5.21 ± 0.57

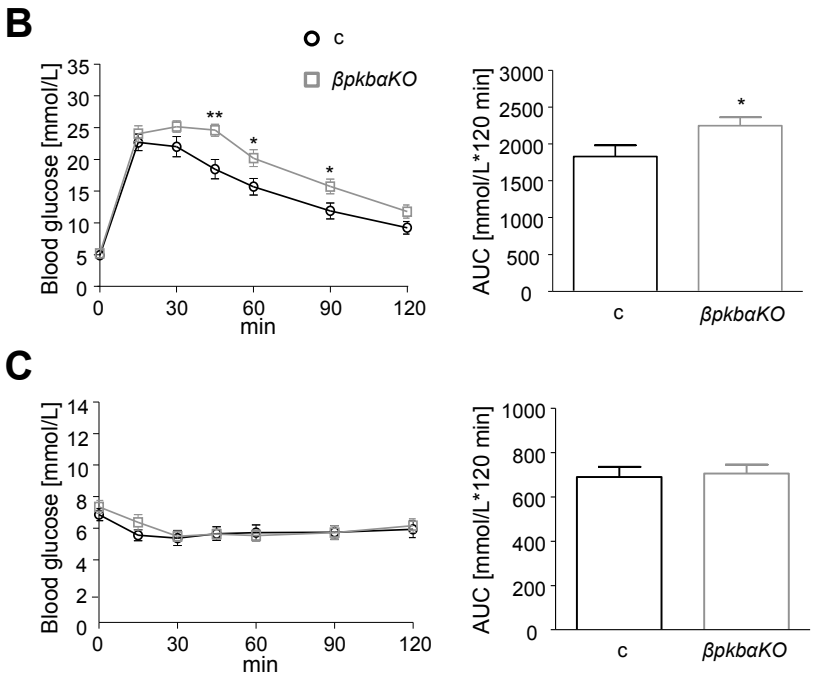


Figure 2. *βpkbαKO* mice show deteriorated glucose tolerance at the age of 26 weeks.

(A) Body weight, random-fed, and fasting blood glucose levels. Time course up to 120 min of blood glucose concentrations during (B) ipGTT and (C) ipITT in *βpkbαKO* mice (grey open squares) and controls (c) (black open circles). The respective AUCs are shown as bar graphs on the right side. Data are presented as mean ± SEM from, n ≥ 9mice. *, $P \leq 0.05$.

A

	control	βpkbaKO
Weight [g]	31.38 ± 0.59	31.2 ± 0.8
Blood glucose [mmol/L]		
random-fed	8.95 ± 0.21	8.59 ± 0.34
3h fasting	9.17 ± 0.33	9.52 ± 0.31
18h fasting	5.28 ± 0.4	5.99 ± 0.56

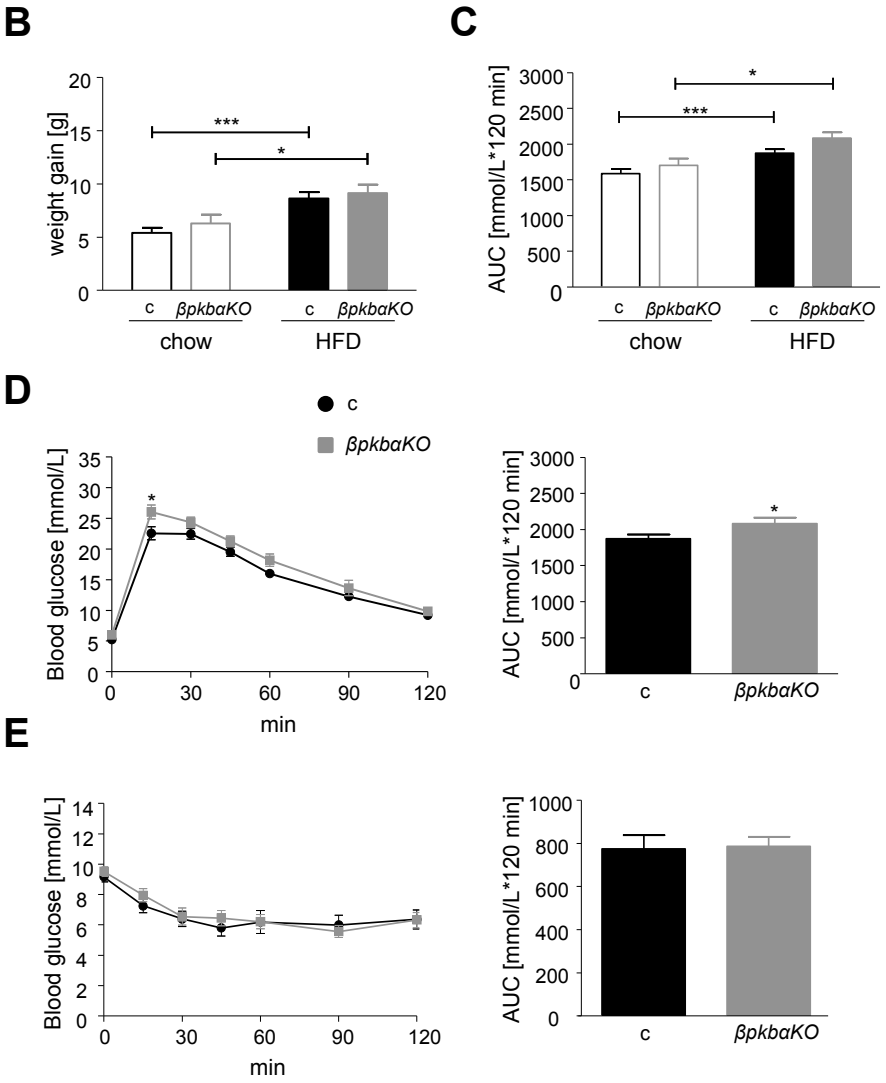


Figure 3. *βpkbaKO* mice show deteriorated glucose tolerance after 6 weeks of HFD.

(A) Body weight, random-fed and fasting blood glucose levels. (B) Weight gain after 6 weeks of HFD. (C) AUC of ipGTT of *βpkbaKO* mice and controls (c) under chow diet (grey open bars and black open bars, respectively) and HFD (grey filled bars and black filled bars, respectively). Time course up to 120 min of blood glucose concentrations in *βpkbaKO* mice (grey filled squares) and controls (c) (black filled circles) during (D) ipGTT and (E) ipITT. The respective AUCs are shown as bar graphs on the right side. Data are presented as mean ± SEM from, n=10 mice. *, $P \leq 0.05$.

A

	control	*βpkbaKO*
Weight [g]	34.8 ± 1.09	33.9 ± 1.15
Blood glucose [mmolL]		
random-fed	8.9 ± 0.4	9.2 ± 0.3
3h fasting	9.36 ± 0.24	9.04 ± 0.36
18h fasting	5.88 ± 0.63	6.2 ± 0.77

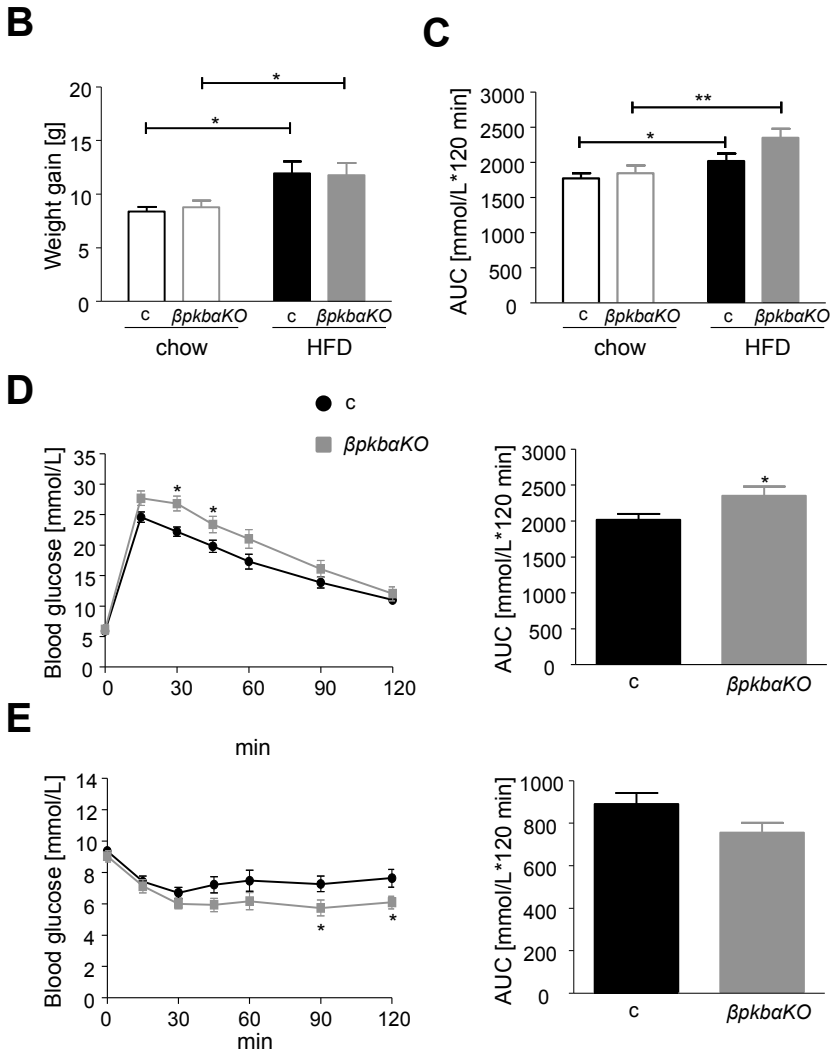


Figure 4. *βpkbaKO* mice show deteriorated glucose tolerance after 12 weeks of HFD.

Body weight, random–fed, and fasting blood glucose levels. (B) Weight gain after 12 weeks of HFD. (C) AUC of ipGTT of *βpkbaKO* mice and controls (c) under chow diet (grey open bars and black open bars, respectively) and HFD (grey filled bars and black filled bars, respectively). Time course up to 120 min of blood glucose concentrations in *βpkbaKO* mice (grey filled squares) and controls (c) (black filled circles) during (D) ipGTT and (E) ipITT. The respective AUCs are shown as bar graphs on the right side. Data are presented as mean ± SEM from, n=10 mice. *, $P \leq 0.05$.

A

	control	*βpkbaKO*
Weight [g]	41.64 ± 2.7	36.9 ± 1.9
Blood glucose [mmolL]		
0h fasting	9.0 ± 0.4	8.9 ± 0.17
3h fasting	8.47 ± 0.56	8.06 ± 0.4
18h fasting	6.22 ± 0.42	6.65 ± 0.28

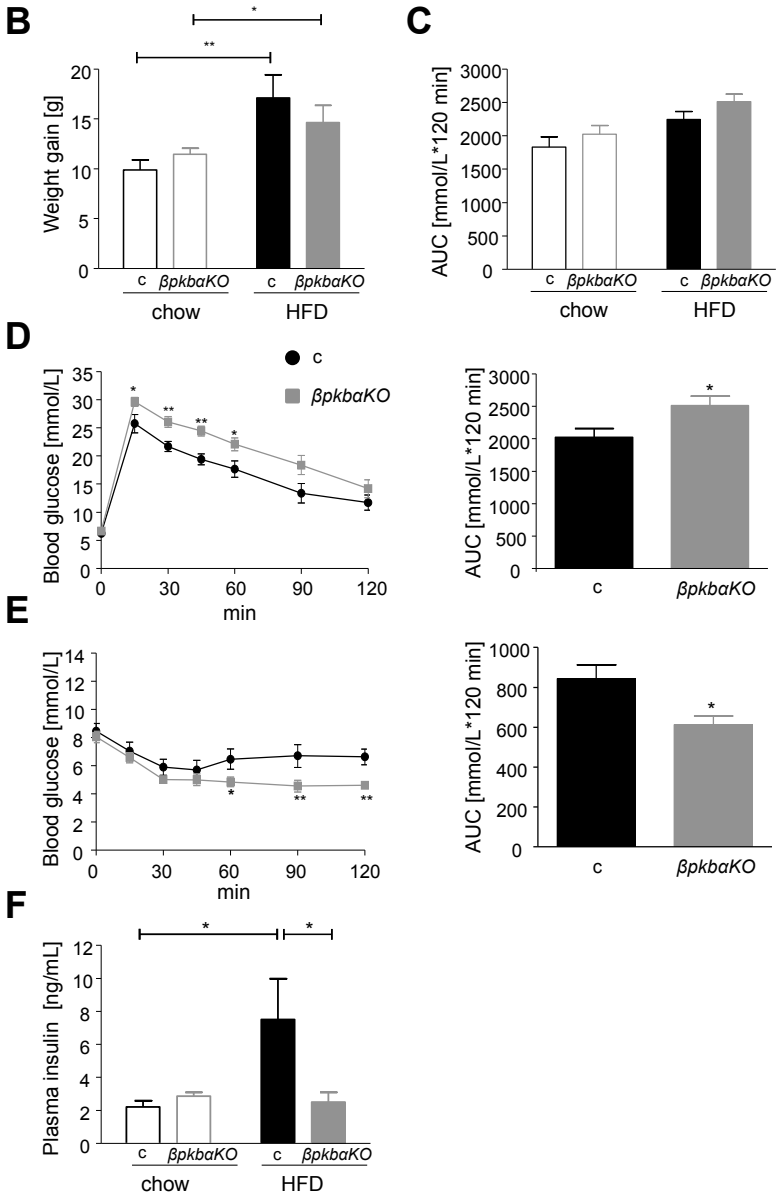


Figure 5. **Deteriorated glucose tolerance but improved insulin sensitivity in *βpkbaKO* mice after 20 weeks of HFD.**

Body weight, random–fed, and fasting blood glucose levels. (B) Weight gain after 20 weeks of HFD. (C) AUC of ipGTT of *βpkbaKO* mice and controls (c) under chow diet (grey open bars and black open bars, respectively) and HFD (grey filled bars and black filled bars, respectively). Time course up to 120 min of blood glucose concentrations in *βpkbaKO* mice (grey filled squares) and controls (c) (black filled circles) during (D) ipGTT and (E) ipITT. The respective AUCs are shown as bar graphs on the right side. (F) Random-fed plasma insulin levels in chow fed *βpkbaKO* mice and controls (c) (grey open bars and black open bars, respectively) and after HFD (grey filled bars and black filled bars, respectively). The data are presented as mean ± SEM, from n ≥ 6 mice. *, $P \leq 0.05$, **, $P \leq 0.01$

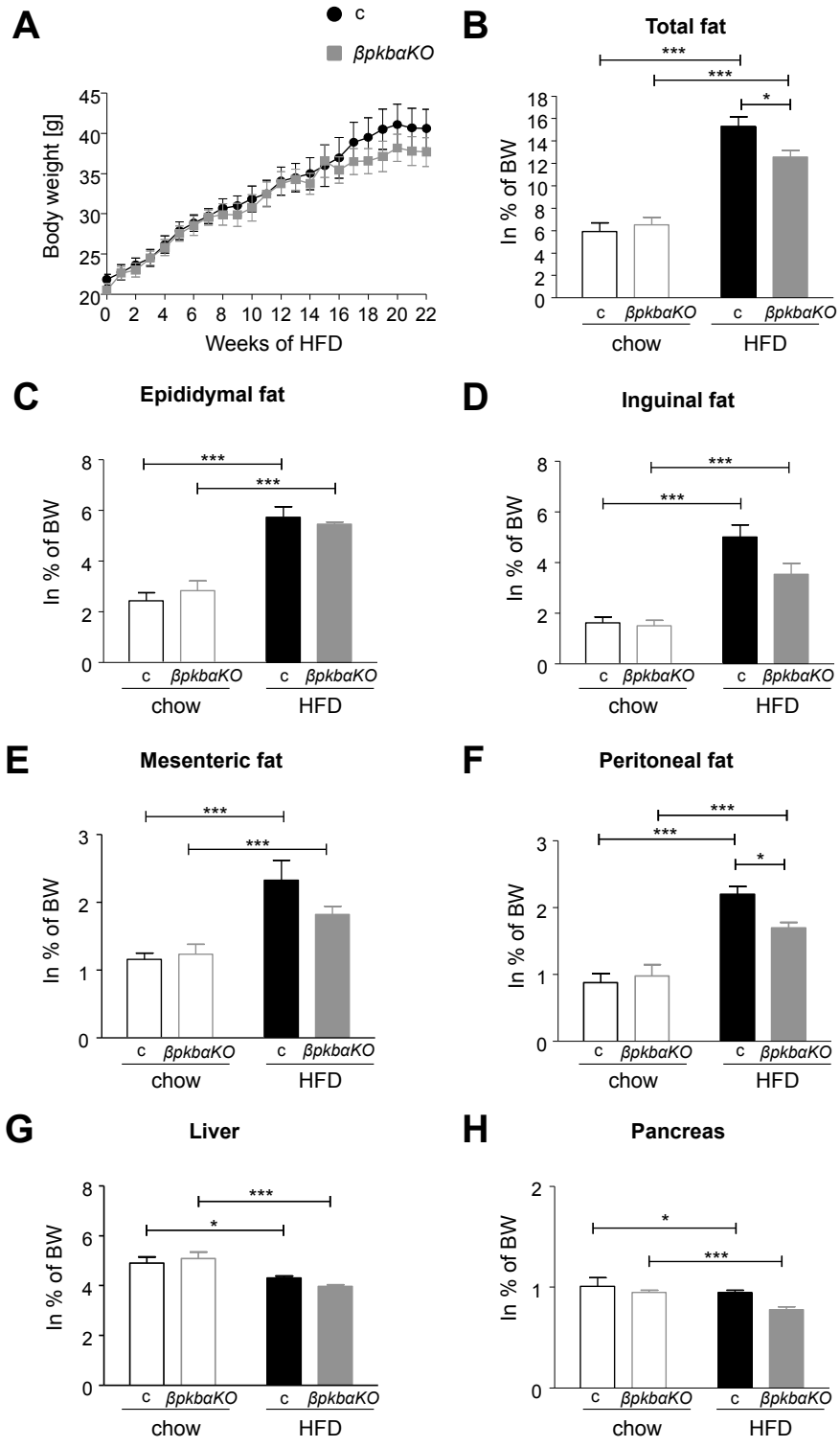


Figure 6. *βpkbαKO* mice show reduced adipose tissue compared to controls.

(A) Weight gain of *βpkbαKO* mice (grey filled squares) and of controls (c) (black filled circles) up to 22 weeks of HFD. Animals were 28 weeks old at the end of the HFD experiment (22 weeks of HFD). They were sacrificed and organs as well as fat from different depots were dissected. Weight of the dissected tissues in % of body weight (BW) for: (B) total fat, (C) epididymal fat, (D) inguinal fat, (E) mesenteric fat, (F) peritoneal fat, (G) liver and (H) pancreas. *βpkbαKO* mice: chow-fed (grey open bars) and HFD-fed (grey filled bars); controls (c): chow-fed (black open bars) and HFD-fed (black filled bars). Data are presented as mean ± SEM, from n ≥ 5 mice. *, $P \leq 0.05$, **, $P \leq 0.01$.

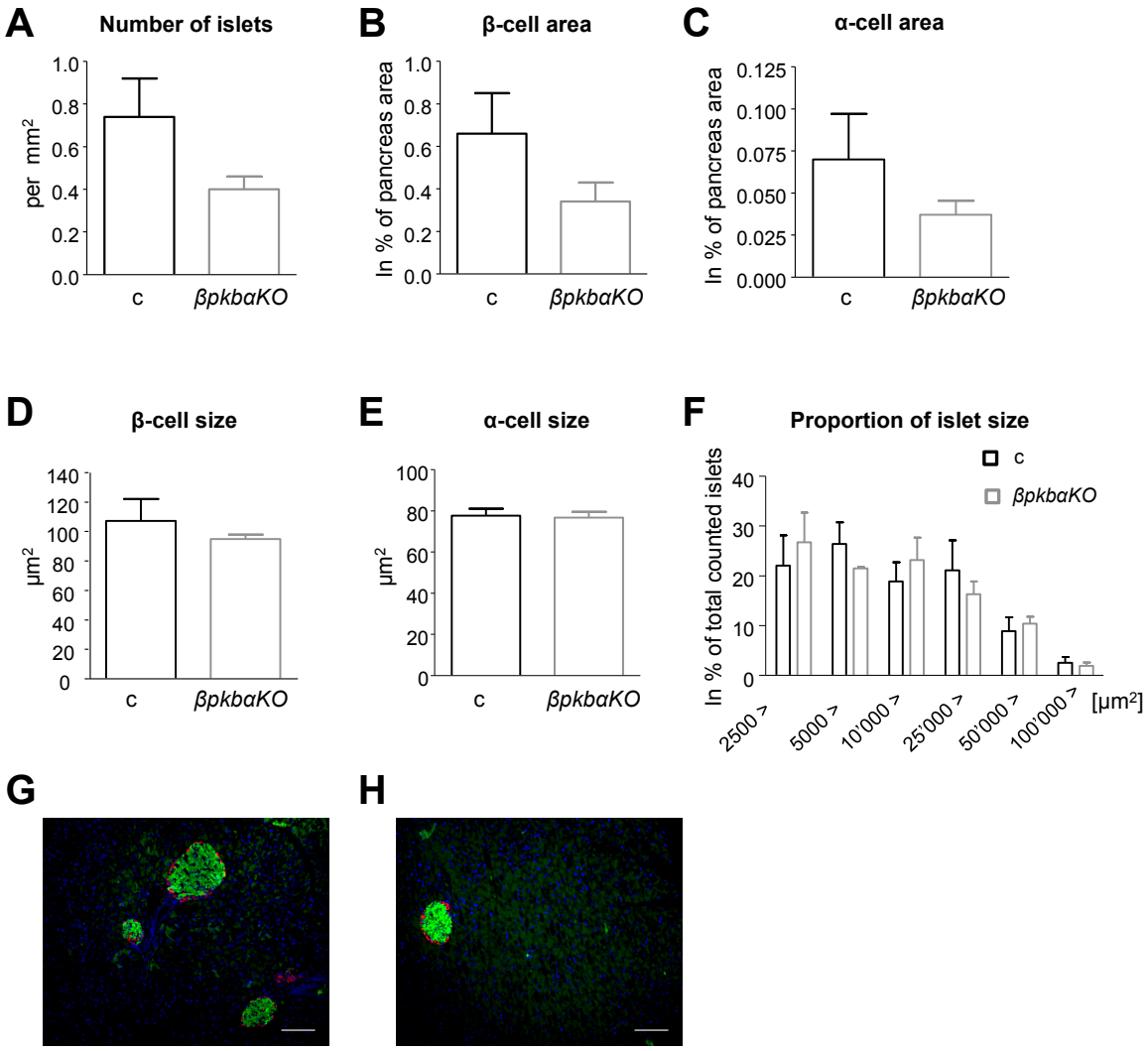
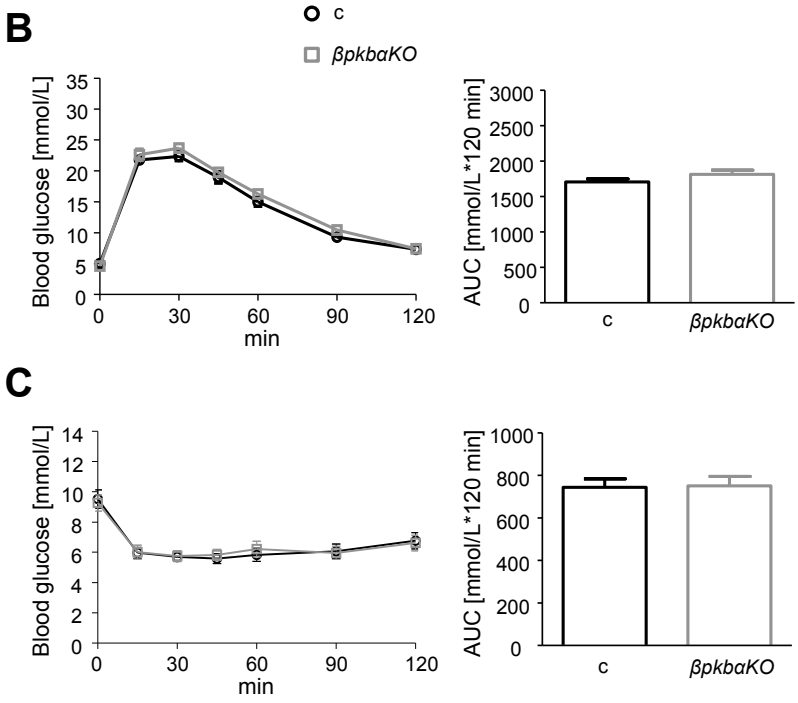


Figure 7. *βpkbαKO* mice show decreased islet area relative to controls.

Animals (28 weeks old) under chow diet were sacrificed, pancreas was dissected and embedded in paraffin. Pancreatic sections were stained for insulin, glucagon, and DAPI to determine (A) islet area, (B) β-cell area, (C) α-cell area, (D) β-cell size, (E) α-cell size and (F) islet size in *βpkbαKO* (grey open bars) and controls (c) (black open bars). Representative microscopic images of (G) controls (c) and (H) *βpkbαKO* stained to visualise insulin (green), glucagon (red) and nuclei (blue). Scale bars, 100 µm. Data are presented as mean ± SEM with n=4 from 28 weeks old chow-fed mice.

A

	control	*βpkbαKO*
Weight [g]	25.1 ± 0.74	25.3 ± 0.8
Blood glucose [mmol/L]		
random-fed	9.5 ± 0.4	9.45 ± 0.33
3h fasting	9.5 ± 0.6	9.27 ± 0.53
18h fasting	4.99 ± 0.18	4.6 ± 0.21

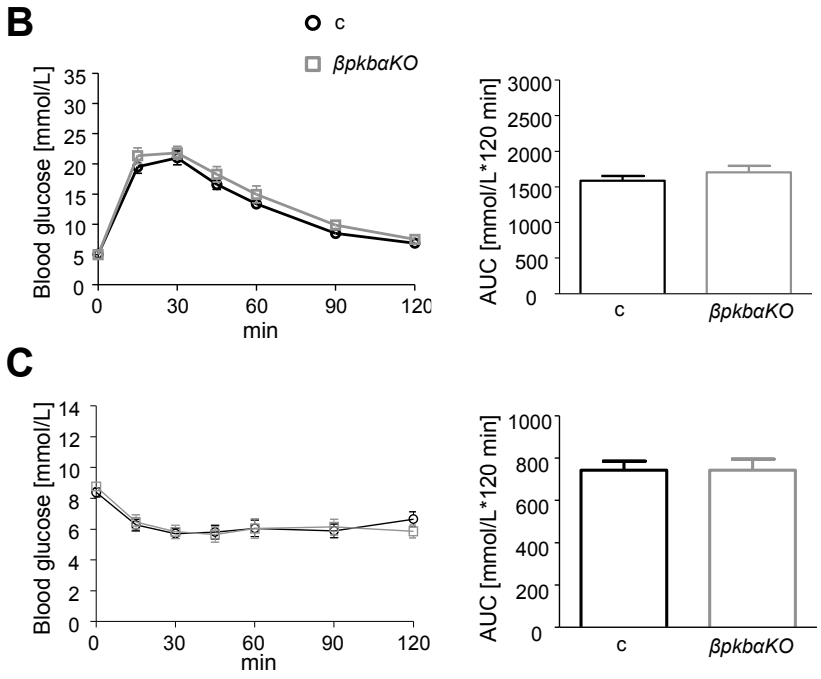


Supplementary figure 1. **Metabolic parameters of 6 weeks old chow-fed mice.**

(A) Body weight, random fed and fasting blood glucose levels. Time course up to 120 min of blood glucose concentrations in *βpkbαKO* mice (grey open squares) and controls (c) (black open circles) during (B) ipGTT and (C) ipITT. The respective AUCs are shown as bar graphs on the right side. Data are presented as mean ± SEM from, n ≥10.

A

	control	*βpkbαKO*
Weight [g]	28.25 ± 0.49	28.4 ± 0.82
Blood glucose [mmol/L]		
random-fed	8.9 ± 0.36	9.2 ± 0.24
3h fasting	8.4 ± 0.3	8.77 ± 0.2
18h fasting	5.02 ± 0.4	4.9 ± 0.3

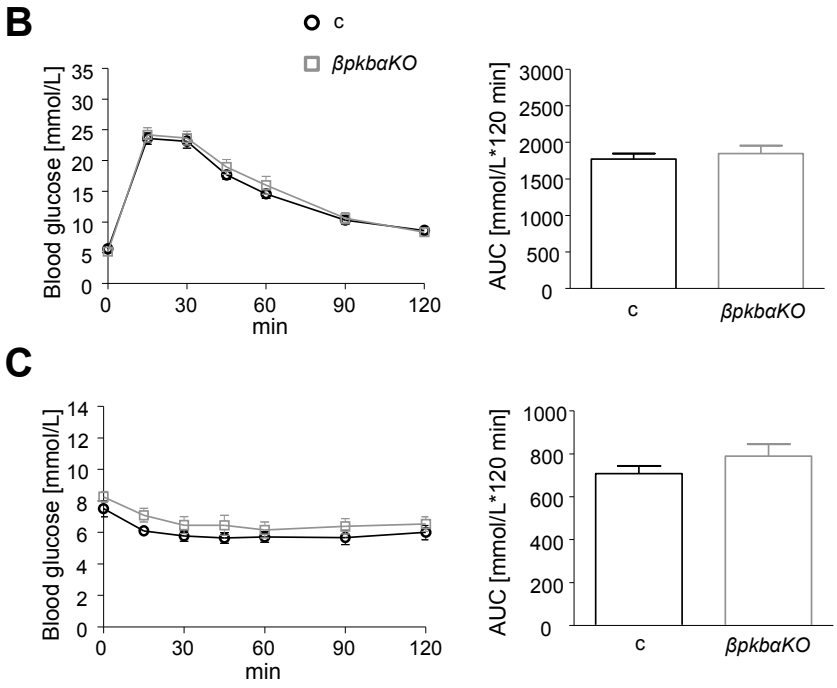


Supplementary figure 2. **Metabolic parameters of 12 weeks old chow-fed mice.**

(A) Body weight, random fed and fasting blood glucose levels. Time course up to 120 min of blood glucose concentrations in *βpkbαKO* mice (grey open squares) and controls (c) (black open circles) during (B) ipGTT and (C) ipITT. The respective AUCs are shown as bar graphs on the right side. Data are presented as mean ± SEM from, n ≥10.

A

	control	*βpkbaKO*
Weight [g]	31.26 ± 0.42	30.87 ± 0.64
Blood glucose [mmolL]		
random-fed	8.9 ± 0.36	8.37 ± 0.31
3h fasting	7.5 ± 0.48	8.26 ± 0.34
18h fasting	5.6 ± 0.17	5.13 ± 0.5



Supplementary figure 3. **Metabolic parameters of 18 weeks old chow-fed mice.**

(A) Body weight, random fed and fasting blood glucose levels. Time course up to 120 min of blood glucose concentrations in *βpkbaKO* mice (grey open squares) and controls (c) (black open circles) during (B) ipGTT and (C) ipITT. The respective AUCs are shown as bar graphs on the right side. Data are presented as mean ± SEM from, n ≥10.

2.2.4 Discussion

IRS2 is an essential survival factor for β-cells as indicated by the severe islet phenotype of mice lacking *Irs2* only in β-cells or in all cells of their body [196]. In contrast, deficiency for *Pkb* isoforms induces relatively mild effects in pancreatic islets [43]. This discrepancy in phenotypes could be due to redundancy in islet functions of PKB isoforms or due to the existence of PKB-independent pathways required downstream for IRS2-dependent regulation of islet mass and function. Previously published work had indicated that PKB isoforms are not only differentially regulated in β-cells, but also perform specific functions [43]. Regulation of compensatory expansion of functional β-cell mass might be isoform-specific. Only overexpression of PKBα in isolated mouse islets increases β-cell proliferation and only PKBα is phosphorylated after overexpression of IRS2 [43].
The aim of this study was to test the role of PKBα in adaptive expansion of functional β-cell mass under HFD-induced insulin resistance.

2.2.4.1 Is *Pkba* required for maintenance and/or adaptive expansion of islet mass?

Male mice lacking *Pkbα* only in β-cells show normal blood glucose and normal glucose tolerance up to the age of 26 weeks. This phenotype suggests that *Pkbα* is not required in β-cells under conditions of normal insulin sensitivity. Older *βpkbαKO* animals with supposedly age-related reduction of insulin sensitivity showed reduced glucose tolerance. Furthermore, under HFD-feeding reduced glucose tolerance compared to littermate controls was observed at a younger age. These observations are in line with the notion that *Pkbα* is required for β-cell compensation under reduced insulin sensitivity but not for maintenance when insulin sensitivity is normal. That *βpkbαKO* animals fail to compensate was also evident from reduced circulating insulin levels in our HFD experiment.
Decreased β-cell area with normal islet size relative to total pancreas area in *βpkbαKO* mice suggests that age-dependent failure to compensate is caused by a reduction in islet number. However, it remains unclear if this is the result of islet loss in adulthood, failure to form new islets or even caused by impaired embryonic development. Furthermore, it remains unclear if β-cell failure is only caused by reduced β-cell mass or also the consequence of impaired β-cell function. These open questions will be addressed in further approaches.
Under HFD feeding *βpkbαKO* mice displayed an increase in insulin sensitivity compared to controls from the age of 18 weeks (12 weeks HFD) on and throughout the rest of the experiment. After 26 weeks (20 weeks HFD), AUC was significant decreased in *βpkbαKO* mice compared to controls. Increased insulin sensitivity in *βpkbαKO* mice might result because of persistently reduced insulin levels. Rodents under HFD show increased phosphorylation of IRS1 at Ser632 and Ser302 [138, 273]. It was suggested that high levels of circulating insulin could be responsible for inhibition of IRS proteins leading to progressive insulin resistance [138, 230, 234, 273]. *βpkbαKO* mice with lower levels of circulating insulin might avoid this negative feed-back regulation.
In a previous study authors expressed a kinase-dead mutant form of PKBα (estimated to reduce PKB activity by 80%) specifically in β-cells. Similar to our findings, mice developed glucose intolerance and

failure to increase plasma insulin levels after HFD-feeding. However, analysis of pancreas morphology showed no alterations in islet composition between mice expressing the kinase-dead variant and wt littermates, neither under chow nor HFD. Nevertheless, a strong insulin secretion defect was observed in these mice [23], possibly due to reduced activation of PKBβ. In contrast, our *βpkbαKO* mice are only deficient for PKBα but showed no change in the expression of PKBβ or PKBγ.

2.2.4.2 The use of *RIP* to drive CRE expression in pancreatic β-cells

We chose *RIP-Cre*^{Herr} for specific deletion of *Pkbα* in pancreatic β-cells. Other studies have used the promoter *pancreatic and duodenal homeobox 1* (*Pdx1*) to drive Cre expression [85, 287], but we suspected that its use might delete *Pkbα* very early in pancreas development possibly resulting in embryonic defects. The *Pdx1* promoter was used to study pancreas respective endocrine cell development [101, 111, 113, 287, 308]. There are different variants of *RIP* promoters available to drive Cre expression. They differ in length of the fragment used and in the genomic insertion site [286]. Other *RIP* promoters (e.g. in *RIP-Cre*^{Mgn}) [213] show robust Cre-mediated recombination in β-cells but also during ventral brain development [86]. Ectopic activation of this *RIP* promoter was found in most brain areas, especially in mid-brain and ventral regions [286]. We chose the strain *RIP-Cre*^{Herr} as it was shown to show minimal ectopic activation making it most suitable for specific β-cell deletion of *Pkbα*. However, it is known that *RIP-Cre*^{Herr} can also be activated in brain, especially in hypothalamus [286]. Activation in these cells was described only as punctual [239], however, the central nervous system (CNS) can control glucose homeostasis also by affecting β-cells. Insulin secretion is also regulated by hypothalamus and insulin can regulate energy homeostasis via the hypothalamus [238]. Formally, it can therefore not be excluded, that the focus of the observed phenotype is not in β-cells. [222, 239, 286].

2.2.4.3 Gender-specific differences

Our study reveals gender-specific differences. First, male mice readily became obese under HFD feeding while females did not. The HFD-induced weight gain in females was insufficient in *βpkbαKO* and in controls. Significantly increased bodyweight and impaired glucose tolerance and insulin sensitivity was only observed after 26 weeks of HFD. Furthermore, no significant increase in total fat was found in HFD-fed females compared to chow-fed females in both groups. All these together indicate that HFD feeding was not efficient in females. Our results fit well to previous observations [264, 265, 296]. To extend the period of HFD in females as proposed by others [264, 265] but also alternative ways to induce insulin resistance should be considered. For example β-cell-specific deficiency for *Pkbα* could be tested in an *ob/ob* (impaired leptin production) or *db/db* (leptin receptor defects) background. Second, only *βpkbαKO* males but not the females developed glucose intolerance. Gender-specific expressivity is not uncommon [173]. Females gained less weight throughout the course of our experiments and might hence retain high insulin sensitivity longer into their adult live. This could postpone the onset of glucose intolerance in *βpkbαKO* females beyond 26 weeks of age.

2.2.4.4 Differences in fat distribution

One can distinguish between intraabdominal (IA) and subcutaneous (SC) white adipose tissue (WAT) in mice. High levels of intraabdominal fat increase the risk for metabolic disorders, whereas the correlation is inverted for subcutaneous fat [141]. Epididymal-, mesenteric-, and peritoneal fat contribute to IA WAT, whereas inguinal fat is associated to SC WAT [229]. The particular fat depots show proteome and morphological differences e.g. size of adipocytes is highest in epididymal fat and lowest in mesenteric- and inguinal fat. Increased adipocyte size is associated with poor metabolic performance in obese individuals [127]. Inguinal fat is in general associated with lower oxidative stress mediated by triglycerides turnover than mesenteric- and epididymal fat [229].

Analysis of fat depots showed significantly decreased total fat relative to bodyweight in *βpkbaKO* mice compared to controls. Mesenteric, inguinal, and peritoneal fat was reduced, whereas epididymal fat was unchanged compared to controls. It is well known that feeding a HFD induces obesity and insulin resistance in rodents. The magnitude of insulin resistance correlates with weight gain and fat accumulation [267, 273]. Increased insulin sensitivity in *βpkbaKO* mice could result from decreased fat mass compared to controls. However, inguinal fat was found to be reduced in *βpkbaKO* mice. Increased inguinal fat mass relative to IA fat is usually associated with improved glucose tolerance in mice [141]. Hence, decreased inguinal fat mass in *βpkbaKO* mice compared to controls might contribute to reduced glucose tolerance in those mice.

βpkbaKO mice were less prone to HFD-induced weight gain at the later time points of HFD. This was associated with a lack of hyperinsulinaemia and increased insulin sensitivity. At this point we can only speculate that the increase in insulin sensitivity does not balance the reduction in insulin and that insufficient insulin activity is the result. Reduced weight gain and fat mass in *βpkbaKO* mice could therefore be the result of reduced plasma insulin levels compared to control mice [181].

Our data suggest that mice with deficiency for *Pkbα* in pancreatic β-cells develop β-cell failure under conditions of insulin resistance. Our data are in line with the previously published model, that predicted PKBα to be only required for adaptation of functional β-cell mass under a challenge but dispensable for its maintenance [196]. In further experiments it should be examined if β-cell failure is the result of islet loss or the result of failure to form new islets in adulthood or even caused by impaired embryonic development. Additionally, β-cell function *in vivo* as well as *in vitro* must be examined. This project is still on going, and further approaches are described in section 4 *(*Future directions).

2.3 Regulation of PKB isoforms in β-cells by growth factors

2.3.1 Introduction

It was an aim of this thesis to study the role of PKB isoforms in the regulation of β-cell growth, proliferation and survival. A number of studies have indicated that the IRS2-PKB axis indeed plays a critical role in β-cells for proliferation, survival, growth, and insulin production [95, 146, 161, 285], In this part we wanted to test how known β-cell growth factors regulate/activate the different PKB isoforms. Insulin and insulin-like growth factor 1 (IGF1) are known inducers of proliferation and activators of PKB in β-cells [60]. Prolactin and glucagon-like peptide (GLP1) are also known to mediate β-cell proliferation, whereas cytokines like interleukin 1β (IL1β) induces β-cell apoptosis via inhibition and ubiquitination of IRS2, which in turn inhibits PKB activation [24, 135, 220].

IGF1 can stimulate β-cell proliferation via activation of the PI3K/PKB axis and inhibition of PKB activity mediated trough ectopic expression of a kinase-dead form of PKB decreases IGF1-induced proliferation [60]. Deficiency of IGFR in β-cells (βIGFRKO) leads to increased fasting insulin levels, moderate glucose intolerance, and to impaired first-phase insulin secretion. Islet mass was normal in these mice [148]. Mice with deficiency in *Ir* are slightly retarded in embryonic growth, develop hyperglycaemia and hyperinsulinaemia and die shortly after birth [5, 129]. Mice with a specific deficiency of IR in β-cells (βIRKO) show similar phenotype like mice with β-cell specific deletion of IGFR [147].

As described above, all three isoforms of PKB (PKBα, β and γ) are regulated at two phosphorylation sites, respectively: 3-phosphoinositide-dependent protein kinase-1 (PDPK1, PDK1) phosphorylates PKB at Thr308 (α: Thr308, β: Thr309, γ: Thr305) accounting for around 10% of kinase activity. Phosphorylation at Ser473 (α: Ser473, β: Ser474, γ: Ser472) via mammalian target of rapamycin complex 2 (mTORC2), DNA-dependent protein kinase (DNA-PK) or ataxia telangiectasia mutated kinase (ATM) leads to full PKB activation. However, it remains unknown if there is isoform-specific regulation by growth factors/inhibitors in β-cells [236, 292].

Specific antibodies are available to detect PKBα, β or γ, but there are no phospho-specific antibodies available to distinguish activation of individual isoforms. The amino acid sequences surrounding the regulated phosphorylation sites are nearly identical. We therefore decided to start our study of isoform-specific activation by Western blotting in combination with isoform-specific immunoprecipitation (IP) in rat insulinoma cells (INS1) as PKB activation had previously been successfully studied in this model [43, 60]. Later we wanted to extend our analysis to isolated rat islets.

It was our goal to first study how PKB isoforms are regulated by insulin and IGF1, two well-defined growth factors, and to later include others, like e.g. D-glucose, the incretin GLP1 and eventually also known β-cell inhibitors (e.g. cytokines or free fatty acids (FFAs)). In the course of this part of the project to many technical limitations became apparent and therefore our analysis of the regulation of PKB isoforms in β-cells was stopped at an early point. Results were nevertheless compiled and are presented in a short form below.

2.3.2 Materials and Methods

2.3.2.1 Cell culture and stimulation with growth factors

Rat insulinoma cells (INS1) [14] from clone E^{110} [187] were cultured on 10 cm plates (TPP Techno Plastic Products, Trasadingen, Switzerland) up to 80% confluence in RPMI medium containing 10.6 mmol/L glucose (Invitrogen, Carlsbad, California, USA), 10% FCS (Hyclone Laboratories Inc., Logan, Utah, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamax, 1 mM sodium pyruvate, 10 mM HEPES, and freshly added 50 µM β-mercaptoethanol (Invitrogen, Carlsbad, California, USA). Cells were starved for 5 hours prior to stimulation with growth factors in medium containing 1.6 mmol/L glucose, 0% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamax, 1 mM sodium pyruvate, 10 mM HEPES, and freshly added 50 µM β-mercaptoethanol (Invitrogen, Carlsbad, California, USA). Bovine insulin and or IGF1 were obtained from Sigma-Aldrich, Saint Louis, Missouri, USA.

2.3.2.2 Immunoprecipitation of PKB isoforms

Cells were washed two times with ice cold PBS and afterwards lysed in lysis buffer containing 50 mM HEPES, 140 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 3 µg/mL leupeptin, 3 µg/mL aprotinin, 10 mM sodium fluoride, 1 mM disodium pyrophosphate and 1 mM sodium orthovanadate. To measure protein concentration in lysates, the Bicinchoninic Acid Assay (BCA Protein Assay Kit, Thermo Scientific, Rockford, USA) was used. Lysates containing 500 µg protein in 300 µl lysis buffer were incubated in the presence of Protein A beads (rpm Protein A Sepharose Fast Flow, GE Healthcare, Buckinghamshire, England) with specific antibodies against PKBα, PKBβ or PKBγ overnight at 4 °C. Antibodies produced in rabbits immunised with isoform-specific peptides [43, 71, 269, 298] were kindly provided by Prof. Brian Hemmings (FMI Basel). After several washing steps, proteins bound to beads were dissociated by two washes (5 minutes per step) at 70°C in NuPAGE LDS Sample Buffer with Sample Reducing Agent (Invitrogen, Carlsbad, California, USA). Eluates were frozen at -20°C for later analysis.

2.3.2.3 Western blotting

The NuPAGE system from Invitrogen was used for Western blotting. In brief, samples were diluted to equal concentrations with NuPAGE LDS Sample Buffer and Sample Reducing Agent (Invitrogen, Carlsbad, California, USA). Equal volumes were loaded onto NuPAGE Novex 4-12% Bis-Tris gradient Gels (Invitrogen, Carlsbad, California, USA). In some experiments and as indicated 9% Tris-glycine SDS-Polyacrylamide gels [150] were used. After electrophoresis, separated proteins were electro-transferred to nitrocellulose membranes (Amersham, GE Healthcare, Buckinghamshire, England). For detection, membranes were incubated overnight at 4°C with first Antibody and for 2h with secondary antibody at room temperature. Between incubations with primary and secondary antibodies, membranes were washed at least five times with TBS-T at room temperature.
Lumi-Light Western Blotting Substrate (Roche, Basel, Switzerland) was used to visualise signals. Signals

were detected and quantified with a LAS-3000 ChemImager (Fuji, Tokyo, Japan) and AIDA software (Raytest, Germany), respectively. Antibodies used for Western blotting are listed in Table 1 (primary antibodies) and Table 2 (secondary antibodies).

Target	Species	Dilution	Catalog N°	Company
phospho-PKB(Ser473)	Mouse	1:1000	4051	Cell Signalling Technology (Beverly, Massachusetts, USA)
phospho-PKB(Thr308)	Rabbit	1:1000	9275	Cell Signalling Technology (Beverly, Massachusetts, USA)
PKBα	Mouse	1:1000	610860	BD Bioscience (Franklin Lakes, New Jersey, USA)
PKBβ	Mouse	1:1000	5239	Cell Signalling Technology (Beverly, Massachusetts, USA)
PKBγ	Rabbit	1:1000	4059	Cell Signalling Technology (Beverly, Massachusetts, USA)

Table1. **Primary antibodies used for Western blotting**

Target	Species	Dilution	Catalog N°	Company
Mouse	Goat	1:3000	sc-2005	Santa Cruz Biotechnnology (Santa Cruz, California, USA)
Rabbit	Goat	1:3000	172-1019	BioRad (Hercules, California, USA)

Table 2. **Secondary antibodies used for Western blotting.**

2.3.2.4 Statistical analysis

Data are shown as mean ± standard error of the mean (SEM) unless indicated otherwise. To test significance of activation after stimulation, unpaired Student's *t*-tests (two-tailed) were performed. For multiple-comparison analysis, normal distribution of data (when $n > 3$) and equality of variances were tested. If variances were not equal, log-transformation was performed. To test significance, balanced one-way ANOVA with Bonferroni's post hoc test was performed. Results were considered to be statistically significant for P values < 0.05 *; $P < 0.001$ **; $P < 0.001$ ***

2.3.3 Results

2.3.3.1 Stimulation with growth factors and immunoprecipitation

In a first approach we assessed the optimal starvation time, concentrations, and duration of stimulation for insulin, IGF1 and for the combined stimulation with insulin and IGF1. Insulin and/or IGF1 were dissolved in 0.1 M $C_2H_4O_2$ and diluted into starvation medium and added to the cells. As control starvation medium containing the same amount like the stimulation medium of 0.1 M $C_2H_4O_2$ was used. Various concentrations and incubation times/conditions for insulin and IGF1 were tested. Strongest induction was observed for insulin and IGF1 when cells had been starved for 5 h and were stimulated at a concentration of 10 nM for 5 minutes at 37°C (data not shown). Hence all experiments were performed under these conditions. Immunoprecipitations were performed according to Buzzi et al., [43].

2.3.3.2 Phosphorylation at Ser473

2.3.3.2.1 IGF1 is a strong activator of PKBα and PKBβ

Stimulation with IGF1 resulted in readily detectable phosphorylation of PKB at Ser473 (Figure 1A, lysates). Immunoprecipitation showed that all isoforms were significant activated by IGF1. PKBβ showed the strongest activation of all isoforms (32.3 ± 2.68 fold, $P \leq 0.001$), followed by PKBα (22.86 ± 4.24 fold, $P \leq 0.01$) and PKBγ (4.22 ± 0.44 fold, $P \leq 0.001$) (Figure 1A, IP eluates).

2.3.3.2.2 Insulin does not activate PKBγ

Stimulation with insulin resulted in far weaker phosphorylation of PKB as compared to IGF1 and no increase in overall PKB phosphorylation without IP was detectable (Figure 1B, lysates). However, differences were detectable after IP: In contrast to IGF1, stimulation with insulin only increased phosphorylation of PKBα (1.98 ± 0.4 fold, n.s) and PKBβ (4.29 ± 0.7 fold, $P \leq 0.01$) but not of PKBγ (0.97 ± 0.098 fold) (Figure 1B, IP eluates).

2.3.3.2.3 Combined stimulation with insulin and IGF1 does not result in additive effects

Combined stimulation with insulin and IGF1 resulted in intermediated overall PKB activation (Figure 1C, lysate) and in altered isoform-specific regulation. Phosphorylation of PKBα was intermediately increased 9.63 ± 4.25 fold, n.s (1.98 ± 0.4 fold and 22.86 ± 4.24 fold for insulin and IGF1 alone, respectively). Stimulation of PKBβ phosphorylation was 29.14 ± 4.8 fold ($P \leq 0.01$) not significantly different from stimulation with IGF1 alone. Phosphorylation of PKBγ was increased 16.47 ± 5.4 fold ($P \leq 0.05$) (Figure 1C, IP eluates), significantly higher compared to stimulation with IGF1 (4.22 ± 0.44 fold) or insulin (0.97 ± 0.098 fold) alone.

2.3.3.3 Phosphorylation at Thr308

2.3.3.3.1 PKBα is not activated at Thr308 after stimulation with IGF1

Stimulation with IGF1 resulted in barely detectable overall PKB activation at Thr308 (not shown). However, weak but analysable signals/differences were obtained after IP. PKBβ was the most and only significant activated isoform after stimulation with IGF1 (3.49 ± 0.06 fold, $P \leq 0.001$) followed by PKBγ (1.7 ± 0.29 fold, n.s). PKBα was not activated (Figure 2). No signal could be detected after stimulation with insulin and combination of insulin and IGF-1, respectively, neither in lysates nor after IP (not shown).

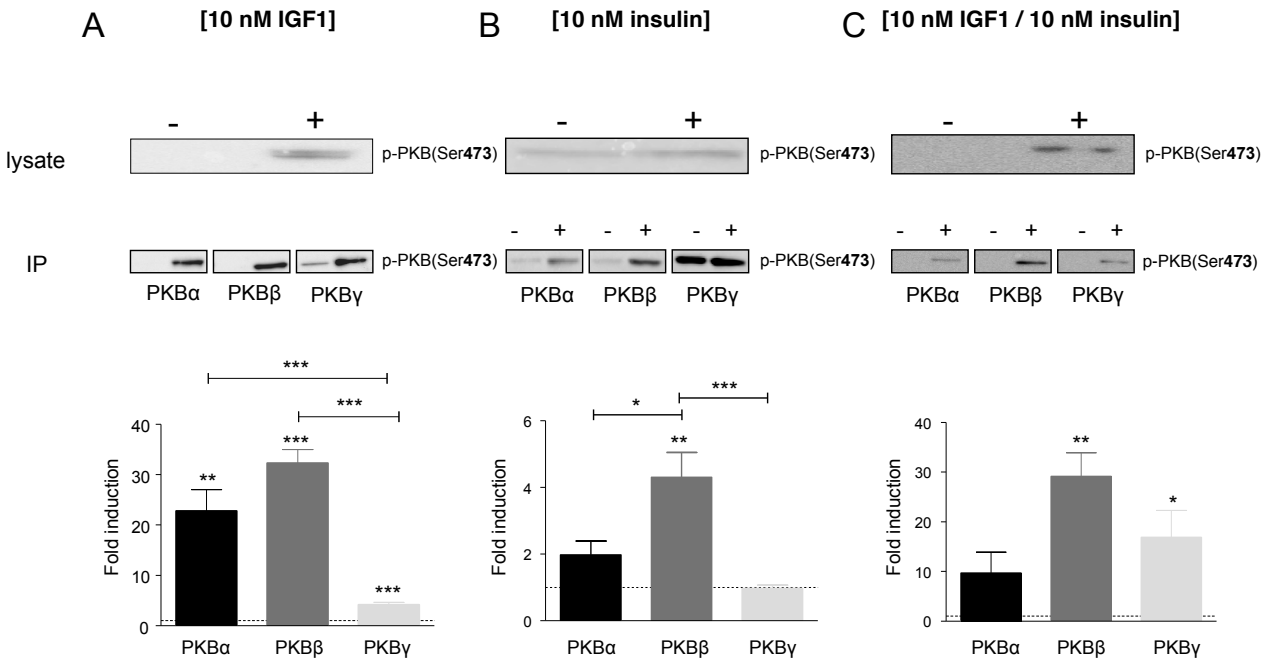


Figure 1. **Isoform-specific activation of PKB at Ser473.**

INS1 cells were stimulated as indicated for 5 minutes with IGF1, insulin or with a mix of both (A, B, and C, respectively). Total PKB phosphorylation at Ser473 was assessed in lysates (top row of immunoblots). Immunoprecipitation of PKB isoforms allowed detection of isoform-specific phosphorylation at Ser473 (lower row of immunoblots). Representative immunoblots are shown. After quantification of signals, fold induction relative to unstimulated condition was calculated for each isoform and mean values are shown in bar graphs. Data are presented as mean ± SEM (n ≥ 3). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

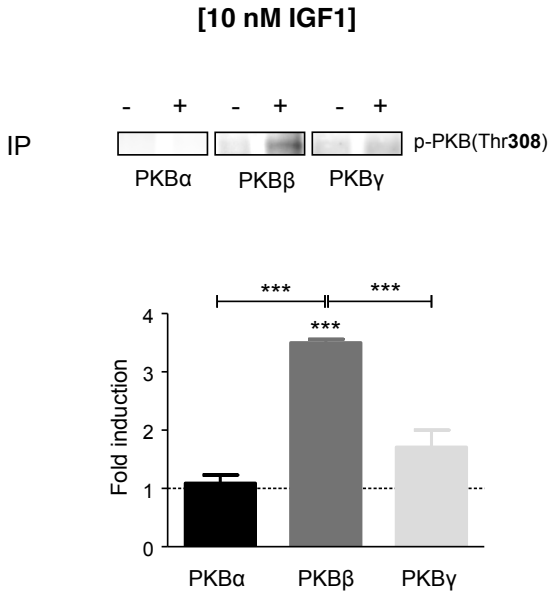


Figure 2. **Isoform-specific activation of PKB at Thr308 after IGF1 stimulation.**

INS1 cells were stimulated for 5 minutes with IGF1. Immunoprecipitation of PKB isoforms allowed detection of isoform-specific phosphorylation at Thr308. A representative immunoblot is shown (n=3). After quantification of signals, fold induction relative to unstimulated condition was calculated for each isoform and mean values are shown in bar graphs. Data are presented as mean ± SEM (n=3). *** $P \leq 0.001$.

2.3.4. Discussion

In β-cells, insulin, IGF1 and other growth factors/inhibitors are considered to control β-cell mass and function via regulation of PKB. However, three isoforms of PKB exist and it remains unknown, which isoform(s) are/is activated/inhibited after stimulation with stimulators and inhibitors. Our study was an attempt to evaluate isoform-specific phosphorylation at Ser473 and Thr308, respectively, after stimulation with insulin, IGF1 or after combined stimulation with insulin and IGF1.

Obtained results suggest that regulation of PKB isoforms is highly complex even with insulin and IGF1, two ligands supposedly activating very similar intra-cellular signal transduction pathways. Quantitative differences in activation of PKB isoforms at Ser473 and Thr308 after stimulation with IGF1, insulin or after combined stimulation with both were observed. Overall, IGF1 stimulated phosphorylation of PKB more compared to insulin. This difference in potency might depend on different expression levels of the IR and the IGFR in INS1. Under all conditions tested, PKBβ showed the highest increase in phosphorylation at Ser473 and Thr308 compared to the other two isoforms, but the extent of activation was still variable depending on the respective stimulation condition. IGF1 stimulated phosphorylation of PKBβ at Ser473 more than seven times stronger compared to insulin. Deficiency for IGFR, IR or PKBβ results in insulin secretion defects [23, 147, 148] and hence regulation of PKBβ by IGF and insulin might be required to regulate this important β-cell function.

PKBα was regulated by insulin and IGF1 at Ser473 but not at Thr308. Interestingly, combined stimulation with insulin and IGF1 was not synergistic but resulted in intermediate levels of phosphorylation. To the best of our knowledge such an effect has not been described previously. It could be caused by competition of receptors for different substrates, but there is no evidence available to confirm or dismiss this possibility.

In line with previously published results [43] we found significant basal phosphorylation of PKBγ at Ser473 (Figure 2B, IP eluates). Phosphorylation of PKBγ at Ser473 and Thr308 was slightly induced by IGF1 but not by insulin. However, combined stimulation with insulin and IGF1 potentiated the increase in phosphorylation at Ser473, but not at Thr308.

Such complex patterns with synergistic and antagonistic activation are difficult to interpret. Possibly, not the regulation of a single isoform determines activation of downstream targets but their relative activation. If this was true, isoform-specific activation of PKB, including differential regulation of the two phosphorylation sites, could integrate over different signals; in such a case correlation of PKB activation and downstream function(s) would be difficult to assess.

Analysis of phosphorylation at Thr308 proved to be difficult as only small increases could be detected after stimulation with IGF1. Possible explanations for this observation are: There is no significant regulation of Thr308 in INS1 cells under the chosen conditions and/or the affinity of the antibody is to low. Unfortunately, a better antibody to detect phospho-PKB(Thr308) could not be obtained. Also due to this technical difficulty and in light of the high degree of complexity of the regulation of PKB isoforms it was decided to stop this part of the project.

Further approaches should include detailed analysis on all levels: assessment of the activation of PKB isoforms, analysis of the activation of downstream targets of PKB and studies of the biological effects such as proliferation, apoptosis and β-cell growth. To apply mass spectrometry possibly in combination with immunoprecipitations might help to increase sensitivity and validity of quantifications.

3. Conclusions and general discussion

The starting point of this thesis was the striking discrepancy in severity of islet phenotypes between *Irs2* deficient mice and mice deficient for isoforms of *Pkb*. The broader aim of this thesis was to define the underlying mechanisms and to test if previous findings regarding the functions of PKBα and PKBβ in rodents are transferrable to humans. PKB isoforms in β-cells were studied in three different approaches: In a first approach (section 2.1) we induced GOF for PKBα and PKBβ in human islets by transfection with adenoviral expression constructs (results shown in Table 2). Results strongly suggest that PKBα is the isoform that can increase human β-cell mass via the induction of proliferation and survival. Overexpression of PKBα but not of PKBβ increased the number of β-cells relative to non-β-cells significantly. Overexpression of neither PKBα nor PKBβ affected β-cell function.
Our new data are well in line with findings in rodent β-cells and this suggests a high degree of evolutionary conservation of the functions of the IRS2-PKB axis in the regulation of islet mass.

		Proliferation	Apoptosis		β-cell size
			Basal	IL1β-induced	
Intact islets		α	-	α	α & β
Dispersed islets	Non-β-cells	α	α & β	n/a	-
	β-cells	α & β	α	n/a	α & β

Table 2. **Summary of GOF effects for PKBα and PKBβ in human islets.**

In the second approach (sections 2.2), the role of PKBα was studied in a newly generated insulin resistant conditional LOF model with specific deficiency for *Pkbα* in β-cells (*βpkbαKO*). The goal of this approach was to test if PKBα is required only for compensatory expansion of β-cell mass but not for maintenance, as was previously proposed (model shown in Figure 3) [196]. This question could not be addressed in existing mouse models with whole body deficiency for *Pkbα* because they show increased peripheral insulin sensitivity. The new model was successfully generated and results obtained are in line with the notion that PKBα is only required for compensatory expansion of β-cell mass but dispensable for maintenance. Younger animals showed normal glucose tolerance but aged and insulin resistant animals showed reduced glucose tolerance. However, we cannot exclude at this point that functions of PKB isoforms are redundant in young but non-redundant in older animals. Furthermore, islet mass should be studied in young (new born) animals lacking *Pkbα* in β-cells to test for possible developmental defects. These open questions are currently under investigation.

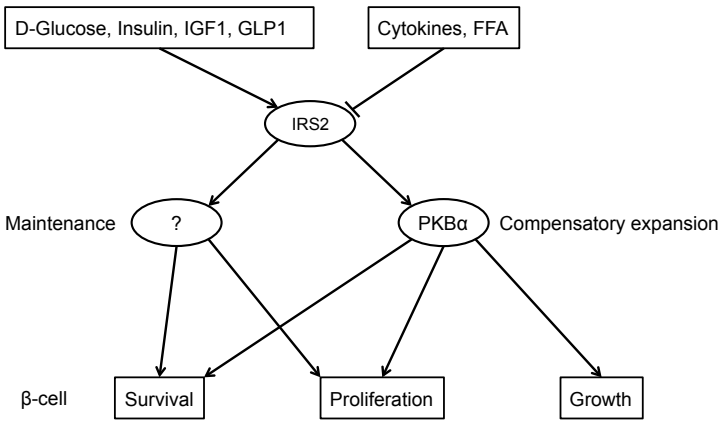


Figure 3. **Hypothetical model describing the regulation of plasticity of islet mass** [196].

In the third part of my project I studied the regulation/activation of PKB isoforms by insulin and IGF1, both well-defined growth factors for β-cells. The aim was to link biological functions in β-cells such as GSIS, proliferation, cellular growth and survival to individual isoforms. Unfortunately, it soon became clear that the taken experimental approach was inappropriate to reach the aim and it was decided to discontinue this part of the project. Nevertheless, obtained results indicate that PKB isoforms can be differentially activated, not only in an isoform-specific manner but also at the two regulated phosphorylation sites (Ser473 and Thr308), in line with the notion that isoforms can have specific roles in β-cells. To the best of our knowledge there is no conclusive published information available at this point regarding a possible underlying molecular mechanism.

During the last decade, research on the pathogenesis of β-cell failure in type 2 diabetes has become a major focus. Currently available therapeutic drugs improve glucose homeostasis via increasing insulin sensitivity (e.g. glitazone), enhancing insulin secretion (e.g. sulfonylurea antidiabetics), reducing absorption (e.g. α-glucosidase inhibitors) or increasing excretion of glucose (SGlT2 inhibitors), respectively. GLP1 receptor agonists improve insulin release and inhibit food uptake via regulation of appetite in the CNS via bromocriptine a dopamine agonist (reviewed in [131]). Recently, a study tested the effect of the IL1β inhibitors Anakinra and Canakinumab in T1D patients. IL1β is increased in patients with T1D as well as in patients with T2D. Both inhibitors did not slow β-cell decline in progressive T1D [192]. However, another study tested the outcome of Canakinumab in metformin-treated patients with T2D. A decrease of HbA1c was observed after such combined treatment possibly due to increased β-cell function [110]. GLP1 agonists might stimulate β-cell proliferation via the IRS2-PKB axis and the EGFR-pathway [125, 144]. However, no effect of liraglutide on human β-cell proliferation in *vitro* could be found [228]. Results presented in this thesis together with previously published observations [25, 43], strongly suggest that PKBα can regulate β-cell proliferation, survival, and size and that it can induce the compensatory expansion of β-cell mass *in vivo* under obesity- and age-dependent decrease of insulin sensitivity. In contrast, high insulin sensitivity in liver, muscle and adipose tissue might require low activation of PKBα [43, 280] but high activation of PKBβ. Future diabetes therapies targeting PKB should take such

tissue/isoform-specific functions into account, also in light of a possible role of PKB activation in cancer development [236].

As PKB isoforms are expressed in most tissues and can regulate many cellular processes any global therapeutic modulation of PKB activation could be associated with severe side effects. Indeed, side effects caused by drugs targeting PKB in cancer treatment support this view. The PI3KT/PKB axis is hyperactivated in various cancers probably ensuring cellular growth and increased survival [77]. Various inhibitors that influence the PI3K/PKB-mTor pathway are already used or are been tested. The substances Everolismus and Tensirolismus, both are inhibitors of the mTOR pathway, support renal cell carcinoma therapy but patients suffer from strong metabolic side effects, such as hyperglycaemia, hypercholesterolaemia, and hyperlipidaemia [77, 186, 236]. Isoform-specific interventions in metabolic diseases as well as in cancer could be a possibility for optimising therapies and to avoid side effects. Prospective success will depend on detailed knowledge of how individual isoforms of PKB function in different tissues. Further investigation focused on tissue specific functions of PKB isoforms and their regulation must be performed. Increased activation of PKBα restricted to β-cells could be achieved by targeting viral expression constructs exclusively to β-cells, development of trans-membrane carriers for β-cell-specific drug uptake, or by antibody-mediated drug delivery [236].

PKB is regulated and appears to function in a complex tissue-/isoform-specific manner. Currently available phospho-specific antibodies to detect activation of PKB do not distinguish between different isoforms. They are hence not suitable to study “quality” (isoform-specificity) in activation of PKB. Their wide-spread use in the study of PKB-dependent signal transduction by Western blotting without prior immunoprecipitation of isoforms should therefore be reconsidered. For example, analysis of phosphorylation of PKB at Ser473 is widely performed to assess insulin sensitivity, however, given that PKB isoforms can perform different functions in insulin sensitive tissues such an analysis has clear limitations. New isoform-specific antibodies to detect PKB activation (or alternative methods such as optimised MassSpec protocols) are required.

4. Future directions

A current focus is to finalise the characterisation of the newly generated mouse model described in results section 2.2. In order to distinguish defects in compensatory increase or impaired maintenance of functional β-cell mass, pancreas morphology will be studied in mice at different age and nutritional status. In addition to islet-, β-cell, α-cell area and size, also β-cell apoptosis and proliferation will be analysed. Dependent on the cause of β-cell decline, markers for different stages of postnatal pancreas development can be included in the analysis. The transcription factor *Ngn3* e.g. could be studied to assess differentiation of pancreatic progenitors into endocrine cells. β-cell-specific maturation could be evaluated by studying the three transcriptions factors *NeuroD/Beta2*, *MafA* and *Pdx1* [225].
Islet function will also be studied. Impaired insulin secretion could also be the reason for β-cell failure in aged or obese *βpkbaKO* mice. β-cell function can be tested *in vivo* by injecting glucose and *in vitro* with isolated islets on ECM-coated dishes (GSIS). We already started to test β-cell function *in vivo*, but data are not complete yet. A more exact analysis of insulin secretion can be achieved with perifusion of islets as this method can also reveal the individual phases of insulin secretion.
HFD-fed *βpkbaKO* mice show increased insulin sensitivity after 20 weeks of HFD. Tissue specific analysis of insulin sensitivity will be performed with different experimental approaches. Insulin-stimulated glucose uptake will be assessed in isolated adipocytes. Furthermore, hyperinsulinemic-euglycemic clamps will be performed to analyse insulin sensitivity in muscle as well as in liver.
Also the underlying signalling pathways of β-cell failure must be elucidated. To this end, expression levels and regulation of downstream targets such as S6K, BAD and AS160 could be studied in β-cells isolated from *βpkbaKO* mice.

5. Abbreviations

4EBP	eIF4E-binding protein
ADA	American Diabetes Association
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AS160	AKT substrate 160kD
ATM	ataxia telangiectasia mutated kinase
ATP	adenosine triphosphate
AUC	area under the curve
BCA	bicinchoninic acid assay
BMI	body mass index
BrdU	5-bromo-2'-deoxyuridine
BW	body weight
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CNS	central nervous system
CREB	cAMP response element binding protein
DAG	diacyl glycerol
DAPI	4', 6-diamidin-2-phenylindol
DNA-PK	DNA-dependent protein kinase
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
ER	endoplasmatic reticulum
FCS	fetal calf serum
FFA	free fatty acids
FOXO	forkhead-O transcriptions factor
G6Pase	glucose-6-phosphatase
GABA	γ-aminobutyric acid
GH	growth hormon
GLP	glucagon-like-peptide
GLUT	glucose transporter
GOF	gain of function
GPR	G-protein coupled receptor
Grb2	growth factor receptor-bound protein 2

GSIS	glucose-stimulated insulin secretion
GSK	glycogen synthase kinase
GTT	glucose tolerance test
HA	hemmagglutinin
Hb1AC	glycated hemoglobin
HBSS	hank's balanced salt solution
HFD	high-fat diet
HLA	histocompatibility leukocyte antigen
IA	intraabdominal
IAPP	amyloid polypeptide
IGF	insulin-like growth factor
IGFR	insulin-like-growth factor receptor
IKKβ	IκBα kinase β
IL1β	interleukin 1β
INS	rat insulinoma cells
IP	immunoprecipitation
ip	intraperitoneal
IR	insulin receptors
IRS	insulin receptor substrate
ITT	insulin tolerance test
JNK	c-Jun N-terminal kinase
K_{ATP} channels	ATP-dependent-K^+-channels
KO	knock out
KRB	krebs-Ringer bicarbonate buffer
LOF	loss of function
LPL	lipoprotein lipase
LPS	receptor for endotoxin
MAPK	Ras-mitogen-activated protein kinase
MOI	multiplicities of infection
mTORC	rapamycin complex
NADH	Nicotinamidadenindinukleotid
NFκB	nuclear factor κ-light-chain-enhancer of activated B-cells
NPEY	asparagine-proline glutamic acid phosphotyrosine
PBS	phosphate-buffered saline
PC	pyruvate carboxylase
PDK	kinase 3-phosphoinositide-dependent protein kinase
Pdx	pancreatic and duodenal homeobox
PEPCK	phosphoenolpyruvate carboxykinase 1

PH	NH_2 - terminal pleckstrin homology
PI3K	phosphatidyl inositol 3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
PKB	protein kinase B /Akt
PKC	protein kinase C
PL	placental lactogen
PLC	phospholipase C
PP2A	protein phosphatase 2a
PPAR	peroxisome proliferator-activated receptor γ
Prl	prolactin
PTB	phosphotyrosine-binding
PTEN	tensin homologue
PTPN1, PTP1B	protein tyrosine non-receptor type 1B
RIA	radioimmunoassay
RIP	rat insulin promoter
ROS	radical oxygen species
RP	reserve pools
RRP	readily releasable pool
S6K	ribosomal protein S6
SC	subcutaneous
SD	standard deviation
SEM	standard error of the mean
SH2	Src homology 2
SHC	Src homology domain containing
SHPTP	tyrosine protein phosphatase
T2D	type 2 diabetes
TBS-(T)	tis-buffered saline (tween 20)
TG	triglyceride
THEM, CTMP	thioesterase superfamily members
TK	tyrosine kinase
TLR	toll-like receptor
TNF	tumor necrosis factor
TSC	tuberous sclerosis complex
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
ULK	unc-51-like kinase
UPR	unfolded protein response
VDCC	L-type voltage-dependent Ca^{2+}-channels

VLDL	very-low-density lipoprotein
WAT	white adipose tissue
wt	wild type

6. References

1. The Diabetes Control and Complications Trial Research Group.**The Effect of Intensive Treatment of Diabetes on the Development and Progression of Long-Term Complications in Insulin-Dependent Diabetes Mellitus.** *The New England journal of medicine* 1993, **329**(14):977-986.

2. Uk Prospective Diabetes Study (Ukpds) Group. **Intensive Blood-Glucose Control with Sulphonylureas or Insulin Compared with Conventional Treatment and Risk of Complications in Patients with Type 2 Diabetes (Ukpds 33).** *Lancet* 1998, **352**(9131):837-853.

3. American Diabetes Association (ADA): **Diagnosis and Classification of Diabetes Mellitus**. *Diabetes care* 2009, **32**:Suppl. 1: 62-67.

4. World Health Organization (WHO): **The Top 10 Causes of Death**. *Fact Sheet* 2013, **310**.

5. Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI, Westphal H: **Early Neonatal Death in Mice Homozygous for a Null Allele of the Insulin Receptor Gene**. *Nature genetics* 1996, **12**(1):106-109.

6. Ackermann AM, Gannon M: **Molecular Regulation of Pancreatic Beta-Cell Mass Development, Maintenance, and Expansion**. *Journal of molecular endocrinology* 2007, **38**(1-2):193-206.

7. Ackler S, Ahmad S, Tobias C, Johnson MD, Glazer RI: **Delayed Mammary Gland Involution in Mmtv-Akt1 Transgenic Mice**. *Oncogene* 2002, **21**(2):198-206.

8. Aguirre V, Uchida T, Yenush L, Davis R, White MF: **The C-Jun Nh(2)-Terminal Kinase Promotes Insulin Resistance During Association with Insulin Receptor Substrate-1 and Phosphorylation of Ser(307)**. *The Journal of biological chemistry* 2000, **275**(12):9047-9054.

9. Ahren B: **Regulation of Insulin Secretion by Nerves and Neuropeptides**. *Annals of the Academy of Medicine, Singapore* 1999, **28**(1):99-104.

10. Aizawa T, Komatsu M: **Rab27a: A New Face in Beta Cell Metabolism-Secretion Coupling**. *The Journal of clinical investigation* 2005, **115**(2):227-230.

11. Alberts B JA, Lewis J, Raff M, Roberts K, Walter P: **Mechanism of Cell Communication**. In: *Molecular Biology of the Cell.* vol. 5: Taylor & Francis LTD.; 2007: 879-965.

12. Amacker-Francoys I, Mohanty S, Niessen M, Spinas GA, Trub T: **The Metabolisable Hexoses D-Glucose and D-Mannose Enhance the Expression of Irs-2 but Not of Irs-1 in Pancreatic Beta-Cells**. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association* 2005, **113**(8):423-429.

13. Araki E, Lipes MA, Patti ME, Bruning JC, Haag B, 3rd, Johnson RS, Kahn CR: **Alternative Pathway of Insulin Signalling in Mice with Targeted Disruption of the Irs-1 Gene**. *Nature* 1994, **372**(6502):186-190.

14. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB: **Establishment of 2-Mercaptoethanol-Dependent Differentiated Insulin-Secreting Cell Lines**. *Endocrinology* 1992, **130**(1):167-178.

15. Authier F, Desbuquois B: **Glucagon Receptors**. *Cellular and molecular life sciences : CMLS* 2008, **65**(12):1880-1899.

16. Bae SS, Cho H, Mu J, Birnbaum MJ: **Isoform-Specific Regulation of Insulin-Dependent Glucose Uptake by Akt/Protein Kinase B**. *The Journal of biological chemistry* 2003, **278**(49):49530-49536.

17. Ballian N, Brunicardi FC: **Islet Vasculature as a Regulator of Endocrine Pancreas Function**. *World journal of surgery* 2007, **31**(4):705-714.

18. Ballou LR, Laulederkind SJ, Rosloniec EF, Raghow R: **Ceramide Signalling and the Immune Response**. *Biochimica et biophysica acta* 1996, **1301**(3):273-287.

19. Bansal P, Wang Q: **Insulin as a Physiological Modulator of Glucagon Secretion**. *American journal of physiology Endocrinology and metabolism* 2008, **295**(4):E751-761.

20. Banting FG, Best CH: **The Internal Secretion of the Pancreas. 1922**. *The Indian journal of medical research* 2007, **125**(3):251-266.

21. Baum J, Simons BE, Jr., Unger RH, Madison LL: **Localization of Glucagon in the Alpha Cells in the Pancreatic Islet by Immunofluorescent Technics**. *Diabetes* 1962, **11**:371-374.

22. Bell GI, Santerre RF, Mullenbach GT: **Hamster Preproglucagon Contains the Sequence of Glucagon and Two Related Peptides**. *Nature* 1983, **302**(5910):716-718.

23. Bernal-Mizrachi E, Fatrai S, Johnson JD, Ohsugi M, Otani K, Han Z, Polonsky KS, Permutt MA: **Defective Insulin Secretion and Increased Susceptibility to Experimental Diabetes Are Induced by Reduced Akt Activity in Pancreatic Islet Beta Cells**. *The Journal of clinical investigation* 2004, **114**(7):928-936.

24. Bernal-Mizrachi E, Kulkarni RN, Scott DK, Mauvais-Jarvis F, Stewart AF, Garcia-Ocana A: **Human Beta-Cell Proliferation and Intracellular Signaling Part 2: Still Driving in the Dark without a Road Map**. *Diabetes* 2014, **63**(3):819-831.

25. Bernal-Mizrachi E, Wen W, Stahlhut S, Welling CM, Permutt MA: **Islet Beta Cell Expression of Constitutively Active Akt1/Pkb Alpha Induces Striking Hypertrophy, Hyperplasia, and Hyperinsulinemia**. *The Journal of clinical investigation* 2001, **108**(11):1631-1638.

26. Bhaskar PT, Hay N: **The Two Torcs and Akt**. *Developmental cell* 2007, **12**(4):487-502.

27. Bliss M: **The Discovery of Insulin: The inside Story**. *Publication - American Institute of the History of Pharmacy* 1997, **16**:93-99.

28. Boller S, Joblin BA, Xu L, Item F, Trub T, Boschetti N, Spinas GA, Niessen M: **From Signal Transduction to Signal Interpretation: An Alternative Model for the Molecular Function of Insulin Receptor Substrates**. *Archives of physiology and biochemistry* 2012, **118**(3):148-155.

29. Bonner-Weir S: **Morphological Evidence for Pancreatic Polarity of Beta-Cell within Islets of Langerhans**. *Diabetes* 1988, **37**(5):616-621.

30. Bost F, Aouadi M, Caron L, Even P, Belmonte N, Prot M, Dani C, Hofman P, Pages G, Pouyssegur J *et al*: **The Extracellular Signal-Regulated Kinase Isoform Erk1 Is Specifically Required for in Vitro and in Vivo Adipogenesis**. *Diabetes* 2005, **54**(2):402-411.

31. Boron WF, Boulpaep EL: **Medical Physiology; A Cellular Molecular Approach**, vol. 2; chapt. 51, 2009.

32. Bouzakri K, Zachrisson A, Al-Khalili L, Zhang BB, Koistinen HA, Krook A, Zierath JR: **Sirna-Based Gene Silencing Reveals Specialized Roles of Irs-1/Akt2 and Irs-2/Akt1 in Glucose and Lipid Metabolism in Human Skeletal Muscle**. *Cell metabolism* 2006, **4**(1):89-96.

33. Bozulic L, Hemmings BA: **Pikking on Pkb: Regulation of Pkb Activity by Phosphorylation**. *Current opinion in cell biology* 2009, **21**(2):256-261.

34. Bratanova-Tochkova TK, Cheng H, Daniel S, Gunawardana S, Liu YJ, Mulvaney-Musa J, Schermerhorn T, Straub SG, Yajima H, Sharp GW: **Triggering and Augmentation Mechanisms, Granule Pools, and Biphasic Insulin Secretion**. *Diabetes* 2002, **51 Suppl 1**:S83-90.

35. Brazil DP, Hemmings BA: **Ten Years of Protein Kinase B Signalling: A Hard Akt to Follow**. *Trends in biochemical sciences* 2001, **26**(11):657-664.

36. Brelje TC, Scharp DW, Lacy PE, Ogren L, Talamantes F, Robertson M, Friesen HG, Sorenson RL: **Effect of Homologous Placental Lactogens, Prolactins, and Growth Hormones on Islet B-Cell Division and Insulin Secretion in Rat, Mouse, and Human Islets: Implication for Placental Lactogen Regulation of Islet Function During Pregnancy**. *Endocrinology* 1993, **132**(2):879-887.

37. Brelje TC, Sorenson RL: **Role of Prolactin Versus Growth Hormone on Islet B-Cell Proliferation in Vitro: Implications for Pregnancy**. *Endocrinology* 1991, **128**(1):45-57.

38. Brose N, Rosenmund C: **Move over Protein Kinase C, You've Got Company: Alternative Cellular Effectors of Diacylglycerol and Phorbol Esters**. *Journal of cell science* 2002, **115**(Pt 23):4399-4411.

39. Brownlee M: **A Radical Explanation for Glucose-Induced Beta Cell Dysfunction**. *The Journal of clinical investigation* 2003, **112**(12):1788-1790.

40. Bruning JC, Winnay J, Cheatham B, Kahn CR: **Differential Signaling by Insulin Receptor Substrate 1 (Irs-1) and Irs-2 in Irs-1-Deficient Cells**. *Molecular and cellular biology* 1997, **17**(3):1513-1521.

41. Buteau J, Foisy S, Joly E, Prentki M: **Glucagon-Like Peptide 1 Induces Pancreatic Beta-Cell Proliferation Via Transactivation of the Epidermal Growth Factor Receptor**. *Diabetes* 2003, **52**(1):124-132.

42. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC: **Beta-Cell Deficit and Increased Beta-Cell Apoptosis in Humans with Type 2 Diabetes**. *Diabetes* 2003, **52**(1):102-110.

43. Buzzi F, Xu L, Zuellig RA, Boller SB, Spinas GA, Hynx D, Chang Z, Yang Z, Hemmings BA, Tschopp O *et al*: **Differential Effects of Protein Kinase B/Akt Isoforms on Glucose Homeostasis and Islet Mass**. *Molecular and cellular biology* 2010, **30**(3):601-612.

44. Cai D, Dhe-Paganon S, Melendez PA, Lee J, Shoelson SE: **Two New Substrates in Insulin Signaling, Irs5/Dok4 and Irs6/Dok5**. *The Journal of biological chemistry* 2003, **278**(28):25323-25330.

45. Cerf ME: **Beta Cell Dysfunction and Insulin Resistance**. *Frontiers in endocrinology* 2013, **4**:37.

46. Chen C, Hosokawa H, Bumbalo LM, Leahy JL: **Mechanism of Compensatory Hyperinsulinemia in Normoglycemic Insulin-Resistant Spontaneously Hypertensive Rats. Augmented Enzymatic Activity of Glucokinase in Beta-Cells**. *The Journal of clinical investigation* 1994, **94**(1):399-404.

47. Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K *et al*: **Growth Retardation and Increased Apoptosis in Mice with Homozygous Disruption of the Akt1 Gene**. *Genes & development* 2001, **15**(17):2203-2208.

48. Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, 3rd, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ: **Insulin Resistance and a Diabetes Mellitus-Like Syndrome in Mice Lacking the Protein Kinase Akt2 (Pkb Beta)**. *Science* 2001, **292**(5522):1728-1731.

49. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ: **Akt1/Pkbalpha Is Required for Normal Growth but Dispensable for Maintenance of Glucose Homeostasis in Mice**. *The Journal of biological chemistry* 2001, **276**(42):38349-38352.

50. Christensen M, Bagger JI, Vilsboll T, Knop FK: **The Alpha-Cell as Target for Type 2 Diabetes Therapy**. *The review of diabetic studies : RDS* 2011, **8**(3):369-381.

51. Christensen M, Vedtofte L, Holst JJ, Vilsboll T, Knop FK: **Glucose-Dependent Insulinotropic Polypeptide: A Bifunctional Glucose-Dependent Regulator of Glucagon and Insulin Secretion in Humans**. *Diabetes* 2011, **60**(12):3103-3109.

52. Condorelli G, Drusco A, Stassi G, Bellacosa A, Roncarati R, Iaccarino G, Russo MA, Gu Y, Dalton N, Chung C *et al*: **Akt Induces Enhanced Myocardial Contractility and Cell Size in Vivo in Transgenic Mice**. *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99**(19):12333-12338.

53. Cook SA, Matsui T, Li L, Rosenzweig A: **Transcriptional Effects of Chronic Akt Activation in the Heart**. *The Journal of biological chemistry* 2002, **277**(25):22528-22533.

54. Corkey BE: **Banting Lecture 2011: Hyperinsulinemia: Cause or Consequence?** *Diabetes* 2012, **61**(1):4-13.

55. Daneman D: **Type 1 Diabetes**. *Lancet* 2006, **367**(9513):847-858.

56. de Koning EJ, van den Brand JJ, Mott VL, Charge SB, Hansen BC, Bodkin NL, Morris JF, Clark A: **Macrophages and Pancreatic Islet Amyloidosis**. *Amyloid : the international journal of experimental and clinical investigation : the official journal of the International Society of Amyloidosis* 1998, **5**(4):247-254.

57. Dean PM: **Ultrastructural Morphometry of the Pancreatic -Cell**. *Diabetologia* 1973, **9**(2):115-119.

58. Del Rio G, Procopio M, Bondi M, Marrama P, Menozzi R, Oleandri SE, Grottoli S, Maccario M, Velardo A, Ghigo E: **Cholinergic Enhancement by Pyridostigmine Increases the Insulin Response to Glucose Load in Obese Patients but Not in Normal Subjects**. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity* 1997, **21**(12):1111-1114.

59. Devendra D, Liu E, Eisenbarth GS: **Type 1 Diabetes: Recent Developments**. *Bmj* 2004, **328**(7442):750-754.

60. Dickson LM, Lingohr MK, McCuaig J, Hugl SR, Snow L, Kahn BB, Myers MG, Jr., Rhodes CJ: **Differential Activation of Protein Kinase B and P70(S6)K by Glucose and Insulin-Like Growth Factor 1 in Pancreatic Beta-Cells (Ins-1)**. *The Journal of biological chemistry* 2001, **276**(24):21110-21120.

61. Donath MY, Boni-Schnetzler M, Ellingsgaard H, Ehses JA: **Islet Inflammation Impairs the Pancreatic Beta-Cell in Type 2 Diabetes**. *Physiology* 2009, **24**:325-331.

62. Donath MY, Ehses JA, Maedler K, Schumann DM, Ellingsgaard H, Eppler E, Reinecke M: **Mechanisms of Beta-Cell Death in Type 2 Diabetes**. *Diabetes* 2005, **54 Suppl 2**:S108-113.

63. Donath MY, Storling J, Maedler K, Mandrup-Poulsen T: **Inflammatory Mediators and Islet Beta-Cell Failure: A Link between Type 1 and Type 2 Diabetes**. *Journal of molecular medicine* 2003, **81**(8):455-470.

64. Dong XC, Copps KD, Guo S, Li Y, Kollipara R, DePinho RA, White MF: **Inactivation of Hepatic Foxo1 by Insulin Signaling Is Required for Adaptive Nutrient Homeostasis and Endocrine Growth Regulation**. *Cell metabolism* 2008, **8**(1):65-76.

65. Drozdowski L, Thomson AB: **Intestinal Hormones and Growth Factors: Effects on the Small Intestine**. *World journal of gastroenterology : WJG* 2009, **15**(4):385-406.

66. Drucker DJ: **The Biology of Incretin Hormones**. *Cell metabolism* 2006, **3**(3):153-165.

67. Drucker DJ, DeForest L, Brubaker PL: **Intestinal Response to Growth Factors Administered Alone or in Combination with Human [Gly2]Glucagon-Like Peptide 2**. *The American journal of physiology* 1997, **273**(6 Pt 1):G1252-1262.

68. Du K, Herzig S, Kulkarni RN, Montminy M: **Trb3: A Tribbles Homolog That Inhibits Akt/Pkb Activation by Insulin in Liver**. *Science* 2003, **300**(5625):1574-1577.

69. Du X, Matsumura T, Edelstein D, Rossetti L, Zsengeller Z, Szabo C, Brownlee M: **Inhibition of Gapdh Activity by Poly(Adp-Ribose) Polymerase Activates Three Major Pathways of Hyperglycemic Damage in Endothelial Cells**. *The Journal of clinical investigation* 2003, **112**(7):1049-1057.

70. Duan C, Xu Q: **Roles of Insulin-Like Growth Factor (Igf) Binding Proteins in Regulating Igf Actions**. *General and comparative endocrinology* 2005, **142**(1-2):44-52.

71. Dummler B, Tschopp O, Hynx D, Yang ZZ, Dirnhofer S, Hemmings BA: **Life with a Single Isoform of Akt: Mice Lacking Akt2 and Akt3 Are Viable but Display Impaired Glucose Homeostasis and Growth Deficiencies**. *Molecular and cellular biology* 2006, **26**(21):8042-8051.

72. Dupont J, Holzenberger M: **Biology of Insulin-Like Growth Factors in Development**. *Birth defects research Part C, Embryo today : reviews* 2003, **69**(4):257-271.

73. Dupont J, LeRoith D: **Insulin and Insulin-Like Growth Factor I Receptors: Similarities and Differences in Signal Transduction**. *Hormone research* 2001, **55 Suppl 2**:22-26.

74. Dutta S, Bonner-Weir S, Montminy M, Wright C: **Regulatory Factor Linked to Late-Onset Diabetes?** *Nature* 1998, **392**(6676):560.

75. Easton RM, Cho H, Roovers K, Shineman DW, Mizrahi M, Forman MS, Lee VM, Szabolcs M, de Jong R, Oltersdorf T *et al*: **Role for Akt3/Protein Kinase Bgamma in Attainment of Normal Brain Size**. *Molecular and cellular biology* 2005, **25**(5):1869-1878.

76. Edelstein AAN, Hoover K, Vale R, Stuurman N: **Computer Control of Microscopes Using Mmanager**. *Current Protocols in Molecular Biology 2010* 2010.

77. Engelman JA: **Targeting Pi3k Signalling in Cancer: Opportunities, Challenges and Limitations**. *Nature reviews Cancer* 2009, **9**(8):550-562.

78. Evans JL, Maddux BA, Goldfine ID: **The Molecular Basis for Oxidative Stress-Induced Insulin Resistance**. *Antioxidants & redox signaling* 2005, **7**(7-8):1040-1052.

79. Fantin VR, Wang Q, Lienhard GE, Keller SR: **Mice Lacking Insulin Receptor Substrate 4 Exhibit Mild Defects in Growth, Reproduction, and Glucose Homeostasis**. *American journal of physiology Endocrinology and metabolism* 2000, **278**(1):E127-133.

80. Federation ID: *IDF Diabetes Atlas* 2013, **6**:7.

81. Feng J, Park J, Cron P, Hess D, Hemmings BA: **Identification of a Pkb/Akt Hydrophobic Motif Ser-473 Kinase as DNA-Dependent Protein Kinase**. *The Journal of biological chemistry* 2004, **279**(39):41189-41196.

82. Forlenza GP, Rewers M: **The Epidemic of Type 1 Diabetes: What Is It Telling Us?** *Current opinion in endocrinology, diabetes, and obesity* 2011, **18**(4):248-251.

83. Frasca F, Pandini G, Sciacca L, Pezzino V, Squatrito S, Belfiore A, Vigneri R: **The Role of Insulin Receptors and Igf-I Receptors in Cancer and Other Diseases**. *Archives of physiology and biochemistry* 2008, **114**(1):23-37.

84. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I: **Increased Oxidative Stress in Obesity and Its Impact on Metabolic Syndrome**. *The Journal of clinical investigation* 2004, **114**(12):1752-1761.

85. Gannon M, Gamer LW, Wright CV: **Regulatory Regions Driving Developmental and Tissue-Specific Expression of the Essential Pancreatic Gene Pdx1**. *Developmental biology* 2001, **238**(1):185-201.

86. Gannon M, Shiota C, Postic C, Wright CV, Magnuson M: **Analysis of the Cre-Mediated Recombination Driven by Rat Insulin Promoter in Embryonic and Adult Mouse Pancreas**. *Genesis* 2000, **26**(2):139-142.

87. Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, Ye J: **Serine Phosphorylation of Insulin Receptor Substrate 1 by Inhibitor Kappa B Kinase Complex**. *The Journal of biological chemistry* 2002, **277**(50):48115-48121.

88. Gao Z, Zhang X, Zuberi A, Hwang D, Quon MJ, Lefevre M, Ye J: **Inhibition of Insulin Sensitivity by Free Fatty Acids Requires Activation of Multiple Serine Kinases in 3t3-L1 Adipocytes**. *Molecular endocrinology* 2004, **18**(8):2024-2034.

89. Garofalo RS, Orena SJ, Rafidi K, Torchia AJ, Stock JL, Hildebrandt AL, Coskran T, Black SC, Brees DJ, Wicks JR *et al*: **Severe Diabetes, Age-Dependent Loss of Adipose Tissue, and Mild Growth Deficiency in Mice Lacking Akt2/Pkb Beta**. *The Journal of clinical investigation* 2003, **112**(2):197-208.

90. Gazzano H, Halban P, Prentki M, Ballotti R, Brandenburg D, Fehlmann M, Van Obberghen E: **Identification of Functional Insulin Receptors on Membranes from an Insulin-Producing Cell Line (Rinm5f)**. *The Biochemical journal* 1985, **226**(3):867-872.

91. Gerich JE: **Physiology of Glucose Homeostasis**. *Diabetes, obesity & metabolism* 2000, **2**(6):345-350.

92. Gianani R: **Beta Cell Regeneration in Human Pancreas**. *Seminars in immunopathology* 2011, **33**(1):23-27.

93. Giovannone B, Scaldaferri ML, Federici M, Porzio O, Lauro D, Fusco A, Sbraccia P, Borboni P, Lauro R, Sesti G: **Insulin Receptor Substrate (Irs) Transduction System: Distinct and Overlapping Signaling Potential**. *Diabetes/metabolism research and reviews* 2000, **16**(6):434-441.

94. Godoy-Matos AF: **The Role of Glucagon on Type 2 Diabetes at a Glance**. *Diabetology & metabolic syndrome* 2014, **6**(1):91.

95. Goldfine AB, Kulkarni RN: **Modulation of Beta-Cell Function: A Translational Journey from the Bench to the Bedside**. *Diabetes, obesity & metabolism* 2012, **14 Suppl 3**:152-160.

96. Gotoh M, Maki T, Kiyoizumi T, Satomi S, Monaco AP: **An Improved Method for Isolation of Mouse Pancreatic Islets**. *Transplantation* 1985, **40**(4):437-438.

97. Greene EL, Lu G, Zhang D, Egan BM: **Signaling Events Mediating the Additive Effects of Oleic Acid and Angiotensin Ii on Vascular Smooth Muscle Cell Migration**. *Hypertension* 2001, **37**(2):308-312.

98. Grodsky GM: **A Threshold Distribution Hypothesis for Packet Storage of Insulin. Ii. Effect of Calcium**. *Diabetes* 1972, **21**(2 Suppl):584-593.

99. Gromada J, Franklin I, Wollheim CB: **Alpha-Cells of the Endocrine Pancreas: 35 Years of Research but the Enigma Remains**. *Endocrine reviews* 2007, **28**(1):84-116.

100. Grossman SP: **The Role of Glucose, Insulin and Glucagon in the Regulation of Food Intake and Body Weight**. *Neuroscience and biobehavioral reviews* 1986, **10**(3):295-315.

101. Gu G, Dubauskaite J, Melton DA: **Direct Evidence for the Pancreatic Lineage: Ngn3+ Cells Are Islet Progenitors and Are Distinct from Duct Progenitors**. *Development* 2002, **129**(10):2447-2457.

102. Guo S, Copps KD, Dong X, Park S, Cheng Z, Pocai A, Rossetti L, Sajan M, Farese RV, White MF: **The Irs1 Branch of the Insulin Signaling Cascade Plays a Dominant Role in Hepatic Nutrient Homeostasis**. *Molecular and cellular biology* 2009, **29**(18):5070-5083.

103. Hakonen E, Ustinov J, Mathijs I, Palgi J, Bouwens L, Miettinen PJ, Otonkoski T: **Epidermal Growth Factor (Egf)-Receptor Signalling Is Needed for Murine Beta Cell Mass Expansion in Response to High-Fat Diet and Pregnancy but Not after Pancreatic Duct Ligation**. *Diabetologia* 2011, **54**(7):1735-1743.

104. Halberg N, Wernstedt-Asterholm I, Scherer PE: **The Adipocyte as an Endocrine Cell**. *Endocrinology and metabolism clinics of North America* 2008, **37**(3):753-768, x-xi.

105. Hanada M, Feng J, Hemmings BA: **Structure, Regulation and Function of Pkb/Akt--a Major Therapeutic Target**. *Biochimica et biophysica acta* 2004, **1697**(1-2):3-16.

106. Harbeck MC, Louie DC, Howland J, Wolf BA, Rothenberg PL: **Expression of Insulin Receptor Mrna and Insulin Receptor Substrate 1 in Pancreatic Islet Beta-Cells**. *Diabetes* 1996, **45**(6):711-717.

107. He L, Hou X, Kanel G, Zeng N, Galicia V, Wang Y, Yang J, Wu H, Birnbaum MJ, Stiles BL: **The Critical Role of Akt2 in Hepatic Steatosis Induced by Pten Loss**. *Am J Pathol* 2010, **176**(5):2302-2308.

108. He L, Hou X, Kanel G, Zeng N, Galicia V, Wang Y, Yang J, Wu H, Birnbaum MJ, Stiles BL: **The Critical Role of Akt2 in Hepatic Steatosis Induced by Pten Loss**. *The American journal of pathology* 2010, **176**(5):2302-2308.

109. Hennige AM, Burks DJ, Ozcan U, Kulkarni RN, Ye J, Park S, Schubert M, Fisher TL, Dow MA, Leshan R *et al*: **Upregulation of Insulin Receptor Substrate-2 in Pancreatic Beta Cells Prevents Diabetes**. *The Journal of clinical investigation* 2003, **112**(10):1521-1532.

110. Hensen J, Howard CP, Walter V, Thuren T: **Impact of Interleukin-1beta Antibody (Canakinumab) on Glycaemic Indicators in Patients with Type 2 Diabetes Mellitus: Results of Secondary Endpoints from a Randomized, Placebo-Controlled Trial**. *Diabetes & metabolism* 2013, **39**(6):524-531.

111. Herrera PL: **Adult Insulin- and Glucagon-Producing Cells Differentiate from Two Independent Cell Lineages**. *Development* 2000, **127**(11):2317-2322.

112. Higaki Y, Wojtaszewski JF, Hirshman MF, Withers DJ, Towery H, White MF, Goodyear LJ: **Insulin Receptor Substrate-2 Is Not Necessary for Insulin- and Exercise-Stimulated Glucose Transport in Skeletal Muscle**. *The Journal of biological chemistry* 1999, **274**(30):20791-20795.

113. Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA *et al*: **Preinvasive and Invasive Ductal Pancreatic Cancer and Its Early Detection in the Mouse**. *Cancer cell* 2003, **4**(6):437-450.

114. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS: **A Central Role for Jnk in Obesity and Insulin Resistance**. *Nature* 2002, **420**(6913):333-336.

115. Holst LS, Mulder H, Manganiello V, Sundler F, Ahren B, Holm C, Degerman E: **Protein Kinase B Is Expressed in Pancreatic Beta Cells and Activated Upon Stimulation with Insulin-Like Growth Factor I**. *Biochemical and biophysical research communications* 1998, **250**(1):181-186.

116. Huang S, Czech MP: **The Glut4 Glucose Transporter**. *Cell metabolism* 2007, **5**(4):237-252.

117. Huotari MA, Palgi J, Otonkoski T: **Growth Factor-Mediated Proliferation and Differentiation of Insulin-Producing Ins-1 and Rinm5f Cells: Identification of Betacellulin as a Novel Beta-Cell Mitogen**. *Endocrinology* 1998, **139**(4):1494-1499.

118. Hutchinson J, Jin J, Cardiff RD, Woodgett JR, Muller WJ: **Activation of Akt (Protein Kinase B) in Mammary Epithelium Provides a Critical Cell Survival Signal Required for Tumor Progression**. *Molecular and cellular biology* 2001, **21**(6):2203-2212.

119. Hutchinson JN, Jin J, Cardiff RD, Woodgett JR, Muller WJ: **Activation of Akt-1 (Pkb-Alpha) Can Accelerate Erbb-2-Mediated Mammary Tumorigenesis but Suppresses Tumor Invasion**. *Cancer research* 2004, **64**(9):3171-3178.

120. Hutley L, Prins JB: **Fat as an Endocrine Organ: Relationship to the Metabolic Syndrome**. *The American journal of the medical sciences* 2005, **330**(6):280-289.

121. Ito T, Sasaki Y, Wands JR: **Overexpression of Human Insulin Receptor Substrate 1 Induces Cellular Transformation with Activation of Mitogen-Activated Protein Kinases**. *Molecular and cellular biology* 1996, **16**(3):943-951.

122. Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H *et al*: **Free Fatty Acids Regulate Insulin Secretion from Pancreatic Beta Cells through Gpr40**. *Nature* 2003, **422**(6928):173-176.

123. Izumi T, Yokota-Hashimoto H, Zhao S, Wang J, Halban PA, Takeuchi T: **Dominant Negative Pathogenesis by Mutant Proinsulin in the Akita Diabetic Mouse**. *Diabetes* 2003, **52**(2):409-416.

124. Jetton TL, Lausier J, LaRock K, Trotman WE, Larmie B, Habibovic A, Peshavaria M, Leahy JL: **Mechanisms of Compensatory Beta-Cell Growth in Insulin-Resistant Rats: Roles of Akt Kinase**. *Diabetes* 2005, **54**(8):2294-2304.

125. Jhala US, Canettieri G, Screaton RA, Kulkarni RN, Krajewski S, Reed J, Walker J, Lin X, White M, Montminy M: **Camp Promotes Pancreatic Beta-Cell Survival Via Creb-Mediated Induction of Irs2**. *Genes & development* 2003, **17**(13):1575-1580.

126. Jiang ZY, Zhou QL, Coleman KA, Chouinard M, Boese Q, Czech MP: **Insulin Signaling through Akt/Protein Kinase B Analyzed by Small Interfering Rna-Mediated Gene Silencing**. *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100**(13):7569-7574.

127. Joe AW, Yi L, Even Y, Vogl AW, Rossi FM: **Depot-Specific Differences in Adipogenic Progenitor Abundance and Proliferative Response to High-Fat Diet**. *Stem cells* 2009, **27**(10):2563-2570.

128. Jones RG, Elford AR, Parsons MJ, Wu L, Krawczyk CM, Yeh WC, Hakem R, Rottapel R, Woodgett JR, Ohashi PS: **Cd28-Dependent Activation of Protein Kinase B/Akt Blocks Fas-Mediated Apoptosis by Preventing Death-Inducing Signaling Complex Assembly**. *The Journal of experimental medicine* 2002, **196**(3):335-348.

129. Joshi RL, Lamothe B, Cordonnier N, Mesbah K, Monthioux E, Jami J, Bucchini D: **Targeted Disruption of the Insulin Receptor Gene in the Mouse Results in Neonatal Lethality**. *The EMBO journal* 1996, **15**(7):1542-1547.

130. Kaburagi Y, Satoh S, Tamemoto H, Yamamoto-Honda R, Tobe K, Veki K, Yamauchi T, Kono-Sugita E, Sekihara H, Aizawa S *et al*: **Role of Insulin Receptor Substrate-1 and Pp60 in the Regulation of Insulin-Induced Glucose Transport and Glut4 Translocation in Primary Adipocytes**. *The Journal of biological chemistry* 1997, **272**(41):25839-25844.

131. Kahn SE, Cooper ME, Del Prato S: **Pathophysiology and Treatment of Type 2 Diabetes: Perspectives on the Past, Present, and Future**. *Lancet* 2014, **383**(9922):1068-1083.

132. Kaiser N, Corcos AP, Sarel I, Cerasi E: **Monolayer Culture of Adult Rat Pancreatic Islets on Extracellular Matrix: Modulation of B-Cell Function by Chronic Exposure to High Glucose**. *Endocrinology* 1991, **129**(4):2067-2076.

133. Kaiser N, Nesher R, Donath MY, Fraenkel M, Behar V, Magnan C, Ktorza A, Cerasi E, Leibowitz G: **Psammomys Obesus, a Model for Environment-Gene Interactions in Type 2 Diabetes**. *Diabetes* 2005, **54 Suppl 2**:S137-144.

134. Kanety H, Feinstein R, Papa MZ, Hemi R, Karasik A: **Tumor Necrosis Factor Alpha-Induced Phosphorylation of Insulin Receptor Substrate-1 (Irs-1). Possible Mechanism for Suppression of Insulin-Stimulated Tyrosine Phosphorylation of Irs-1**. *The Journal of biological chemistry* 1995, **270**(40):23780-23784.

135. Karnik SK, Chen H, McLean GW, Heit JJ, Gu X, Zhang AY, Fontaine M, Yen MH, Kim SK: **Menin Controls Growth of Pancreatic Beta-Cells in Pregnant Mice and Promotes Gestational Diabetes Mellitus**. *Science* 2007, **318**(5851):806-809.

136. Katz LE, Bhala A, Camron E, Nunn SE, Hintz RL, Cohen P: **Igf-Ii, Igf-Binding Proteins and Igf Receptors in Pancreatic Beta-Cell Lines**. *The Journal of endocrinology* 1997, **152**(3):455-464.

137. Kershaw EE, Flier JS: **Adipose Tissue as an Endocrine Organ**. *The Journal of clinical endocrinology and metabolism* 2004, **89**(6):2548-2556.

138. Khamzina L, Veilleux A, Bergeron S, Marette A: **Increased Activation of the Mammalian Target of Rapamycin Pathway in Liver and Skeletal Muscle of Obese Rats: Possible Involvement in Obesity-Linked Insulin Resistance**. *Endocrinology* 2005, **146**(3):1473-1481.

139. Khan AH, Pessin JE: **Insulin Regulation of Glucose Uptake: A Complex Interplay of Intracellular Signalling Pathways**. *Diabetologia* 2002, **45**(11):1475-1483.

140. Kim A, Miller K, Jo J, Kilimnik G, Wojcik P, Hara M: **Islet Architecture: A Comparative Study**. *Islets* 2009, **1**(2):129-136.

141. Kim JY, van de Wall E, Laplante M, Azzara A, Trujillo ME, Hofmann SM, Schraw T, Durand JL, Li H, Li G *et al*: **Obesity-Associated Improvements in Metabolic Profile through Expansion of Adipose Tissue**. *The Journal of clinical investigation* 2007, **117**(9):2621-2637.

142. Kim YK, Kim SJ, Yatani A, Huang Y, Castelli G, Vatner DE, Liu J, Zhang Q, Diaz G, Zieba R *et al*: **Mechanism of Enhanced Cardiac Function in Mice with Hypertrophy Induced by Overexpressed Akt**. *The Journal of biological chemistry* 2003, **278**(48):47622-47628.

143. Kissau L, Stahl P, Mazitschek R, Giannis A, Waldmann H: **Development of Natural Product-Derived Receptor Tyrosine Kinase Inhibitors Based on Conservation of Protein Domain Fold**. *Journal of medicinal chemistry* 2003, **46**(14):2917-2931.

144. Krakowski ML, Kritzik MR, Jones EM, Krahl T, Lee J, Arnush M, Gu D, Mroczkowski B, Sarvetnick N: **Transgenic Expression of Epidermal Growth Factor and Keratinocyte Growth Factor in Beta-Cells Results in Substantial Morphological Changes**. *The Journal of endocrinology* 1999, **162**(2):167-175.

145. Kubota N, Tobe K, Terauchi Y, Eto K, Yamauchi T, Suzuki R, Tsubamoto Y, Komeda K, Nakano R, Miki H *et al*: **Disruption of Insulin Receptor Substrate 2 Causes Type 2 Diabetes Because of Liver Insulin Resistance and Lack of Compensatory Beta-Cell Hyperplasia**. *Diabetes* 2000, **49**(11):1880-1889.

146. Kulkarni RN: **Insulin Action in the Islet Beta Cell**. *Mechanisms of Insulin Action* 2007, **1**:133-151.

147. Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR: **Tissue-Specific Knockout of the Insulin Receptor in Pancreatic Beta Cells Creates an Insulin Secretory Defect Similar to That in Type 2 Diabetes**. *Cell* 1999, **96**(3):329-339.

148. Kulkarni RN, Holzenberger M, Shih DQ, Ozcan U, Stoffel M, Magnuson MA, Kahn CR: **Beta-Cell-Specific Deletion of the Igf1 Receptor Leads to Hyperinsulinemia and Glucose Intolerance but Does Not Alter Beta-Cell Mass**. *Nature genetics* 2002, **31**(1):111-115.

149. Kulkarni RN, Winnay JN, Daniels M, Bruning JC, Flier SN, Hanahan D, Kahn CR: **Altered Function of Insulin Receptor Substrate-1-Deficient Mouse Islets and Cultured Beta-Cell Lines**. *The Journal of clinical investigation* 1999, **104**(12):R69-75.

150. Laemmli UK: **Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4**. *Nature* 1970, **227**(5259):680-685.

151. Lammert E, Cleaver O, Melton D: **Role of Endothelial Cells in Early Pancreas and Liver Development**. *Mechanisms of development* 2003, **120**(1):59-64.

152. Lau DC, Dhillon B, Yan H, Szmitko PE, Verma S: **Adipokines: Molecular Links between Obesity and Atheroslcerosis**. *American journal of physiology Heart and circulatory physiology* 2005, **288**(5):H2031-2041.

153. Lausier J, Diaz WC, Roskens V, LaRock K, Herzer K, Fong CG, Latour MG, Peshavaria M, Jetton TL: **Vagal Control of Pancreatic Ss-Cell Proliferation**. *American journal of physiology Endocrinology and metabolism* 2010, **299**(5):E786-793.

154. Lavan BE, Fantin VR, Chang ET, Lane WS, Keller SR, Lienhard GE: **A Novel 160-Kda Phosphotyrosine Protein in Insulin-Treated Embryonic Kidney Cells Is a New Member of the Insulin Receptor Substrate Family**. *The Journal of biological chemistry* 1997, **272**(34):21403-21407.

155. Lavan BE, Lane WS, Lienhard GE: **The 60-Kda Phosphotyrosine Protein in Insulin-Treated Adipocytes Is a New Member of the Insulin Receptor Substrate Family**. *The Journal of biological chemistry* 1997, **272**(17):11439-11443.

156. Leahy JL: **Pathogenesis of Type 2 Diabetes Mellitus**. *Archives of medical research* 2005, **36**(3):197-209.

157. Leavens KF, Easton RM, Shulman GI, Previs SF, Birnbaum MJ: **Akt2 Is Required for Hepatic Lipid Accumulation in Models of Insulin Resistance**. *Cell Metabolism* 2009, **10**(5):405-418.

158. Leavens KF, Easton RM, Shulman GI, Previs SF, Birnbaum MJ: **Akt2 Is Required for Hepatic Lipid Accumulation in Models of Insulin Resistance**. *Cell metabolism* 2009, **10**(5):405-418.

159. Lee JY, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, Sizemore N, Hwang DH: **Reciprocal Modulation of Toll-Like Receptor-4 Signaling Pathways Involving Myd88 and Phosphatidylinositol 3-Kinase/Akt by Saturated and Polyunsaturated Fatty Acids**. *The Journal of biological chemistry* 2003, **278**(39):37041-37051.

160. Leech CA, Dzhura I, Chepurny OG, Kang G, Schwede F, Genieser HG, Holz GG: **Molecular Physiology of Glucagon-Like Peptide-1 Insulin Secretagogue Action in Pancreatic Beta Cells**. *Progress in biophysics and molecular biology* 2011, **107**(2):236-247.

161. Leibiger IB, Leibiger B, Berggren PO: **Insulin Signaling in the Pancreatic Beta-Cell**. *Annual review of nutrition* 2008, **28**:233-251.

162. LeRoith D, Smith DO: **Loss of Glucose Homoeostasis: Implications of Basal Glycaemia in Type 2 Diabetes**. *Diabetes, obesity & metabolism* 2003, **5**(5):285-294.

163. Li L, Seno M, Yamada H, Kojima I: **Promotion of Beta-Cell Regeneration by Betacellulin in Ninety Percent-Pancreatectomized Rats**. *Endocrinology* 2001, **142**(12):5379-5385.

164. Lin Y, Berg AH, Iyengar P, Lam TK, Giacca A, Combs TP, Rajala MW, Du X, Rollman B, Li W *et al*: **The Hyperglycemia-Induced Inflammatory Response in Adipocytes: The Role of Reactive Oxygen Species**. *The Journal of biological chemistry* 2005, **280**(6):4617-4626.

165. Linetsky E, Bottino R, Lehmann R, Alejandro R, Inverardi L, Ricordi C: **Improved Human Islet Isolation Using a New Enzyme Blend, Liberase**. *Diabetes* 1997, **46**(7):1120-1123.

166. Liu M, Liu F: **Transcriptional and Post-Translational Regulation of Adiponectin**. *The Biochemical journal* 2010, **425**(1):41-52.

167. Liu YQ, Jetton TL, Leahy JL: **Beta-Cell Adaptation to Insulin Resistance. Increased Pyruvate Carboxylase and Malate-Pyruvate Shuttle Activity in Islets of Nondiabetic Zucker Fatty Rats**. *The Journal of biological chemistry* 2002, **277**(42):39163-39168.

168. Lowell BB, Shulman GI: **Mitochondrial Dysfunction and Type 2 Diabetes**. *Science* 2005, **307**(5708):384-387.

169. Lu G, Greene EL, Nagai T, Egan BM: **Reactive Oxygen Species Are Critical in the Oleic Acid-Mediated Mitogenic Signaling Pathway in Vascular Smooth Muscle Cells**. *Hypertension* 1998, **32**(6):1003-1010.

170. Lu M, Wan M, Leavens KF, Chu Q, Monks BR, Fernandez S, Ahima RS, Ueki K, Kahn CR, Birnbaum MJ: **Insulin Regulates Liver Metabolism in Vivo in the Absence of Hepatic Akt and Foxo1**. *Nature medicine* 2012, **18**(3):388-395.

171. In't Veld P, Marichal M: **The Islets of Langerhans; Microscopic Anatomy of the Human Islets of Langerhans**. *Advances in experimental Medicine and Biology* 2010, **654**:1-21.

172. Maahs DM, West NA, Lawrence JM, Mayer-Davis EJ: **Epidemiology of Type 1 Diabetes**. *Endocrinology and metabolism clinics of North America* 2010, **39**(3):481-497.

173. Macotela Y, Boucher J, Tran TT, Kahn CR: **Sex and Depot Differences in Adipocyte Insulin Sensitivity and Glucose Metabolism**. *Diabetes* 2009, **58**(4):803-812.

174. Maedler K, Spinas GA, Lehmann R, Sergeev P, Weber M, Fontana A, Kaiser N, Donath MY: **Glucose Induces Beta-Cell Apoptosis Via Upregulation of the Fas Receptor in Human Islets**. *Diabetes* 2001, **50**(8):1683-1690.

175. Maira SM, Galetic I, Brazil DP, Kaech S, Ingley E, Thelen M, Hemmings BA: **Carboxyl-Terminal Modulator Protein (Ctmp), a Negative Regulator of Pkb/Akt and V-Akt at the Plasma Membrane**. *Science* 2001, **294**(5541):374-380.

176. Majumder PK, Yeh JJ, George DJ, Febbo PG, Kum J, Xue Q, Bikoff R, Ma H, Kantoff PW, Golub TR *et al*: **Prostate Intraepithelial Neoplasia Induced by Prostate Restricted Akt Activation: The Mpakt Model**. *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100**(13):7841-7846.

177. Malstrom S, Tili E, Kappes D, Ceci JD, Tsichlis PN: **Tumor Induction by an Lck-Myrakt Transgene Is Delayed by Mechanisms Controlling the Size of the Thymus**. *Proceedings of the National Academy of Sciences of the United States of America* 2001, **98**(26):14967-14972.

178. Manning BD, Cantley LC: **Akt/Pkb Signaling: Navigating Downstream**. *Cell* 2007, **129**(7):1261-1274.

179. Matsui T, Li L, Wu JC, Cook SA, Nagoshi T, Picard MH, Liao R, Rosenzweig A: **Phenotypic Spectrum Caused by Transgenic Overexpression of Activated Akt in the Heart**. *The Journal of biological chemistry* 2002, **277**(25):22896-22901.

180. Matsui T, Nagoshi T, Hong EG, Luptak I, Hartil K, Li L, Gorovits N, Charron MJ, Kim JK, Tian R *et al*: **Effects of Chronic Akt Activation on Glucose Uptake in the Heart**. *American journal of physiology Endocrinology and metabolism* 2006, **290**(5):E789-797.

181. Mehran AE, Templeman NM, Brigidi GS, Lim GE, Chu KY, Hu X, Botezelli JD, Asadi A, Hoffman BG, Kieffer TJ *et al*: **Hyperinsulinemia Drives Diet-Induced Obesity Independently of Brain Insulin Production**. *Cell metabolism* 2012, **16**(6):723-737.

182. Melloul D, Marshak S, Cerasi E: **Regulation of Insulin Gene Transcription**. *Diabetologia* 2002, **45**(3):309-326.

183. Melloul D, Tsur A, Zangen D: **Pancreatic Duodenal Homeobox (Pdx-1) in Health and Disease**. *Journal of pediatric endocrinology & metabolism : JPEM* 2002, **15**(9):1461-1472.

184. Meloni AR, DeYoung MB, Lowe C, Parkes DG: **Glp-1 Receptor Activated Insulin Secretion from Pancreatic Beta-Cells: Mechanism and Glucose Dependence**. *Diabetes, obesity & metabolism* 2013, **15**(1):15-27.

185. Mende I, Malstrom S, Tsichlis PN, Vogt PK, Aoki M: **Oncogenic Transformation Induced by Membrane-Targeted Akt2 and Akt3**. *Oncogene* 2001, **20**(32):4419-4423.

186. Mendez-Vidal MJ, Martinez Ortega E, Montesa Pino A, Perez Valderrama B, Viciana R: **Management of Adverse Events of Targeted Therapies in Normal and Special Patients with Metastatic Renal Cell Carcinoma**. *Cancer metastasis reviews* 2012, **31 Suppl 1**:S19-27.

187. Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P: **Glucose Sensitivity and Metabolism-Secretion Coupling Studied During Two-Year Continuous Culture in Ins-1e Insulinoma Cells**. *Endocrinology* 2004, **145**(2):667-678.

188. Miettinen PJ, Huotari M, Koivisto T, Ustinov J, Palgi J, Rasilainen S, Lehtonen E, Keski-Oja J, Otonkoski T: **Impaired Migration and Delayed Differentiation of Pancreatic Islet Cells in Mice Lacking Egf-Receptors**. *Development* 2000, **127**(12):2617-2627.

189. Miettinen PJ, Ustinov J, Ormio P, Gao R, Palgi J, Hakonen E, Juntti-Berggren L, Berggren PO, Otonkoski T: **Downregulation of Egf Receptor Signaling in Pancreatic Islets Causes Diabetes Due to Impaired Postnatal Beta-Cell Growth**. *Diabetes* 2006, **55**(12):3299-3308.

190. Mohanty S, Spinas GA, Maedler K, Zuellig RA, Lehmann R, Donath MY, Trub T, Niessen M: **Overexpression of Irs2 in Isolated Pancreatic Islets Causes Proliferation and Protects Human Beta-Cells from Hyperglycemia-Induced Apoptosis**. *Experimental cell research* 2005, **303**(1):68-78.

191. Montanya E, Nacher V, Biarnes M, Soler J: **Linear Correlation between Beta-Cell Mass and Body Weight Throughout the Lifespan in Lewis Rats: Role of Beta-Cell Hyperplasia and Hypertrophy**. *Diabetes* 2000, **49**(8):1341-1346.

192. Moran A, Bundy B, Becker DJ, DiMeglio LA, Gitelman SE, Goland R, Greenbaum CJ, Herold KC, Marks JB, Raskin P *et al*: **Interleukin-1 Antagonism in Type 1 Diabetes of Recent Onset: Two Multicentre, Randomised, Double-Blind, Placebo-Controlled Trials**. *Lancet* 2013, **381**(9881):1905-1915.

193. Muller D, Huang GC, Amiel S, Jones PM, Persaud SJ: **Identification of Insulin Signaling Elements in Human Beta-Cells: Autocrine Regulation of Insulin Gene Expression**. *Diabetes* 2006, **55**(10):2835-2842.

194. Munoz A, Hu M, Hussain K, Bryan J, Aguilar-Bryan L, Rajan AS: **Regulation of Glucagon Secretion at Low Glucose Concentrations: Evidence for Adenosine Triphosphate-Sensitive Potassium Channel Involvement**. *Endocrinology* 2005, **146**(12):5514-5521.

195. Na SY, Patra A, Scheuring Y, Marx A, Tolaini M, Kioussis D, Hemmings BA, Hunig T, Bommhardt U: **Constitutively Active Protein Kinase B Enhances Lck and Erk Activities and Influences Thymocyte Selection and Activation**. *Journal of immunology* 2003, **171**(3):1285-1296.

196. Niessen M: **On the Role of Irs2 in the Regulation of Functional Beta-Cell Mass**. *Archives of physiology and biochemistry* 2006, **112**(2):65-73.

197. Nolan CJ, Leahy JL, Delghingaro-Augusto V, Moibi J, Soni K, Peyot ML, Fortier M, Guay C, Lamontagne J, Barbeau A *et al*: **Beta Cell Compensation for Insulin Resistance in Zucker Fatty Rats: Increased Lipolysis and Fatty Acid Signalling**. *Diabetologia* 2006, **49**(9):2120-2130.

198. O'Brien TD, Butler PC, Westermark P, Johnson KH: **Islet Amyloid Polypeptide: A Review of Its Biology and Potential Roles in the Pathogenesis of Diabetes Mellitus**. *Veterinary pathology* 1993, **30**(4):317-332.

199. Oh YS, Shin S, Lee YJ, Kim EH, Jun HS: **Betacellulin-Induced Beta Cell Proliferation and Regeneration Is Mediated by Activation of Erbb-1 and Erbb-2 Receptors**. *PloS one* 2011, **6**(8):e23894.

200. Okamoto H, Hribal ML, Lin HV, Bennett WR, Ward A, Accili D: **Role of the Forkhead Protein Foxo1 in Beta Cell Compensation to Insulin Resistance**. *The Journal of clinical investigation* 2006, **116**(3):775-782.

201. Olofsson CS, Gopel SO, Barg S, Galvanovskis J, Ma X, Salehi A, Rorsman P, Eliasson L: **Fast Insulin Secretion Reflects Exocytosis of Docked Granules in Mouse Pancreatic B-Cells**. *Pflugers Archiv : European journal of physiology* 2002, **444**(1-2):43-51.

202. Olson AL, Pessin JE: **Structure, Function, and Regulation of the Mammalian Facilitative Glucose Transporter Gene Family**. *Annual review of nutrition* 1996, **16**:235-256.

203. Orci L: **The Insulin Cell: Its Cellular Environment and How It Processes (Pro)Insulin**. *Diabetes/metabolism reviews* 1986, **2**(1-2):71-106.

204. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH, Hotamisligil GS: **Endoplasmic Reticulum Stress Links Obesity, Insulin Action, and Type 2 Diabetes**. *Science* 2004, **306**(5695):457-461.

205. Parsons JA, Brelje TC, Sorenson RL: **Adaptation of Islets of Langerhans to Pregnancy: Increased Islet Cell Proliferation and Insulin Secretion Correlates with the Onset of Placental Lactogen Secretion**. *Endocrinology* 1992, **130**(3):1459-1466.

206. Patra AK, Na SY, Bommhardt U: **Active Protein Kinase B Regulates Tcr Responsiveness by Modulating Cytoplasmic-Nuclear Localization of Nfat and Nf-Kappa B Proteins**. *Journal of immunology* 2004, **172**(8):4812-4820.

207. Peng XD, Xu PZ, Chen ML, Hahn-Windgassen A, Skeen J, Jacobs J, Sundararajan D, Chen WS, Crawford SE, Coleman KG *et al*: **Dwarfism, Impaired Skin Development, Skeletal Muscle Atrophy, Delayed Bone Development, and Impeded Adipogenesis in Mice Lacking Akt1 and Akt2**. *Genes & development* 2003, **17**(11):1352-1365.

208. Peraldi P, Hotamisligil GS, Buurman WA, White MF, Spiegelman BM: **Tumor Necrosis Factor (Tnf)-Alpha Inhibits Insulin Signaling through Stimulation of the P55 Tnf Receptor and Activation of Sphingomyelinase**. *The Journal of biological chemistry* 1996, **271**(22):13018-13022.

209. Peterson RT, Schreiber SL: **Kinase Phosphorylation: Keeping It All in the Family**. *Current biology : CB* 1999, **9**(14):R521-524.

210. Pirola L, Johnston AM, Van Obberghen E: **Modulation of Insulin Action**. *Diabetologia* 2004, **47**(2):170-184.

211. Plum L, Belgardt BF, Bruning JC: **Central Insulin Action in Energy and Glucose Homeostasis**. *The Journal of clinical investigation* 2006, **116**(7):1761-1766.

212. Poitout V, Robertson RP: **Minireview: Secondary Beta-Cell Failure in Type 2 Diabetes--a Convergence of Glucotoxicity and Lipotoxicity**. *Endocrinology* 2002, **143**(2):339-342.

213. Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, Shelton KD, Lindner J, Cherrington AD, Magnuson MA: **Dual Roles for Glucokinase in Glucose Homeostasis as Determined by Liver and Pancreatic Beta Cell-Specific Gene Knock-Outs Using Cre Recombinase**. *The Journal of biological chemistry* 1999, **274**(1):305-315.

214. Prentki M: **New Insights into Pancreatic Beta-Cell Metabolic Signaling in Insulin Secretion**. *European journal of endocrinology / European Federation of Endocrine Societies* 1996, **134**(3):272-286.

215. Prentki M, Corkey BE: **Are the Beta-Cell Signaling Molecules Malonyl-Coa and Cystolic Long-Chain Acyl-Coa Implicated in Multiple Tissue Defects of Obesity and Niddm?** *Diabetes* 1996, **45**(3):273-283.

216. Prentki M, Joly E, El-Assaad W, Roduit R: **Malonyl-Coa Signaling, Lipid Partitioning, and Glucolipotoxicity: Role in Beta-Cell Adaptation and Failure in the Etiology of Diabetes**. *Diabetes* 2002, **51 Suppl 3**:S405-413.

217. Prentki M, Nolan CJ: **Islet Beta Cell Failure in Type 2 Diabetes**. *The Journal of clinical investigation* 2006, **116**(7):1802-1812.

218. Qi Y, Xu Z, Zhu Q, Thomas C, Kumar R, Feng H, Dostal DE, White MF, Baker KM, Guo S: **Myocardial Loss of Irs1 and Irs2 Causes Heart Failure and Is Controlled by P38alpha Mapk During Insulin Resistance**. *Diabetes* 2013, **62**(11):3887-3900.

219. Rathmell JC, Elstrom RL, Cinalli RM, Thompson CB: **Activated Akt Promotes Increased Resting T Cell Size, Cd28-Independent T Cell Growth, and Development of Autoimmunity and Lymphoma**. *European journal of immunology* 2003, **33**(8):2223-2232.

220. Rhodes CJ: **Type 2 Diabetes-a Matter of Beta-Cell Life and Death?** *Science* 2005, **307**(5708):380-384.

221. Rhodes CJ, White MF: **Molecular Insights into Insulin Action and Secretion**. *European journal of clinical investigation* 2002, **32 Suppl 3**:3-13.

222. Rhodes CJ, White MF, Leahy JL, Kahn SE: **Direct Autocrine Action of Insulin on Beta-Cells: Does It Make Physiological Sense?** *Diabetes* 2013, **62**(7):2157-2163.

223. Robertson R, Zhou H, Zhang T, Harmon JS: **Chronic Oxidative Stress as a Mechanism for Glucose Toxicity of the Beta Cell in Type 2 Diabetes**. *Cell biochemistry and biophysics* 2007, **48**(2-3):139-146.

224. Robertson RP: **Chronic Oxidative Stress as a Central Mechanism for Glucose Toxicity in Pancreatic Islet Beta Cells in Diabetes**. *The Journal of biological chemistry* 2004, **279**(41):42351-42354.

225. Rojas AK, Khoo A, Tejedo JR, Bedoya FJ, Soria B, Martin F: **The Islets of Langerhans; Islet Cell Development**. In: *Advances in experimental Medicine and Biology.* vol. 654; 2010.

226. Rui L, Yuan M, Frantz D, Shoelson S, White MF: **Socs-1 and Socs-3 Block Insulin Signaling by Ubiquitin-Mediated Degradation of Irs1 and Irs2**. *The Journal of biological chemistry* 2002, **277**(44):42394-42398.

227. Rutti S, Ehses JA, Sibler RA, Prazak R, Rohrer L, Georgopoulos S, Meier DT, Niclauss N, Berney T, Donath MY *et al*: **Low- and High-Density Lipoproteins Modulate Function, Apoptosis, and Proliferation of Primary Human and Murine Pancreatic Beta-Cells**. *Endocrinology* 2009, **150**(10):4521-4530.

228. Rutti S, Sauter NS, Bouzakri K, Prazak R, Halban PA, Donath MY: **In Vitro Proliferation of Adult Human Beta-Cells**. *PloS one* 2012, **7**(4):e35801.

229. Sackmann-Sala L, Berryman DE, Munn RD, Lubbers ER, Kopchick JJ: **Heterogeneity among White Adipose Tissue Depots in Male C57bl/6j Mice**. *Obesity* 2012, **20**(1):101-111.

230. Samuel VT, Petersen KF, Shulman GI: **Lipid-Induced Insulin Resistance: Unravelling the Mechanism**. *Lancet* 2010, **375**(9733):2267-2277.

231. Santin I, Eizirik DL: **Candidate Genes for Type 1 Diabetes Modulate Pancreatic Islet Inflammation and Beta-Cell Apoptosis**. *Diabetes, obesity & metabolism* 2013, **15 Suppl 3**:71-81.

232. Sawka-Verhelle D, Tartare-Deckert S, White MF, Van Obberghen E: **Insulin Receptor Substrate-2 Binds to the Insulin Receptor through Its Phosphotyrosine-Binding Domain and through a Newly Identified Domain Comprising Amino Acids 591-786**. *The Journal of biological chemistry* 1996, **271**(11):5980-5983.

233. Scaglia L, Smith FE, Bonner-Weir S: **Apoptosis Contributes to the Involution of Beta Cell Mass in the Post Partum Rat Pancreas**. *Endocrinology* 1995, **136**(12):5461-5468.

234. Schenk S, Saberi M, Olefsky JM: **Insulin Sensitivity: Modulation by Nutrients and Inflammation**. *The Journal of clinical investigation* 2008, **118**(9):2992-3002.

235. Schroder M, Kaufman RJ: **Er Stress and the Unfolded Protein Response**. *Mutation research* 2005, **569**(1-2):29-63.

236. Schultze SM, Hemmings BA, Niessen M, Tschopp O: **Pi3k/Akt, Mapk and Ampk Signalling: Protein Kinases in Glucose Homeostasis**. *Expert reviews in molecular medicine* 2012, **14**:e1.

237. Schultze SM, Jensen J, Hemmings BA, Tschopp O, Niessen M: **Promiscuous Affairs of Pkb/Akt Isoforms in Metabolism**. *Archives of physiology and biochemistry* 2011, **117**(2):70-77.

238. Schwartz MW, Figlewicz DP, Baskin DG, Woods SC, Porte D, Jr.: **Insulin in the Brain: A Hormonal Regulator of Energy Balance**. *Endocrine reviews* 1992, **13**(3):387-414.

239. Schwartz MW, Guyenet SJ, Cirulli V: **The Hypothalamus and Ss-Cell Connection in the Gene-Targeting Era**. *Diabetes* 2010, **59**(12):2991-2993.

240. Schwertfeger KL, McManaman JL, Palmer CA, Neville MC, Anderson SM: **Expression of Constitutively Activated Akt in the Mammary Gland Leads to Excess Lipid Synthesis During Pregnancy and Lactation**. *Journal of lipid research* 2003, **44**(6):1100-1112.

241. Schwertfeger KL, Richert MM, Anderson SM: **Mammary Gland Involution Is Delayed by Activated Akt in Transgenic Mice**. *Molecular endocrinology* 2001, **15**(6):867-881.

242. Shanik MH, Xu Y, Skrha J, Dankner R, Zick Y, Roth J: **Insulin Resistance and Hyperinsulinemia: Is Hyperinsulinemia the Cart or the Horse?** *Diabetes care* 2008, **31 Suppl 2**:S262-268.

243. Shaw LM: **The Insulin Receptor Substrate (Irs) Proteins: At the Intersection of Metabolism and Cancer**. *Cell cycle* 2011, **10**(11):1750-1756.

244. Shioi T, McMullen JR, Kang PM, Douglas PS, Obata T, Franke TF, Cantley LC, Izumo S: **Akt/Protein Kinase B Promotes Organ Growth in Transgenic Mice**. *Molecular and cellular biology* 2002, **22**(8):2799-2809.

245. Siddle K: **Molecular Basis of Signaling Specificity of Insulin and Igf Receptors: Neglected Corners and Recent Advances**. *Frontiers in endocrinology* 2012, **3**:34.

246. Singh P, Alex JM, Bast F: **Insulin Receptor (Ir) and Insulin-Like Growth Factor Receptor 1 (Igf-1r) Signaling Systems: Novel Treatment Strategies for Cancer**. *Medical oncology* 2014, **31**(1):805.

247. Song J, Xu Y, Hu X, Choi B, Tong Q: **Brain Expression of Cre Recombinase Driven by Pancreas-Specific Promoters**. *Genesis* 2010, **48**(11):628-634.

248. Srinivasan S, Bernal-Mizrachi E, Ohsugi M, Permutt MA: **Glucose Promotes Pancreatic Islet Beta-Cell Survival through a Pi 3-Kinase/Akt-Signaling Pathway**. *American journal of physiology Endocrinology and metabolism* 2002, **283**(4):E784-793.

249. Steil GM, Trivedi N, Jonas JC, Hasenkamp WM, Sharma A, Bonner-Weir S, Weir GC: **Adaptation of Beta-Cell Mass to Substrate Oversupply: Enhanced Function with Normal Gene Expression**. *American journal of physiology Endocrinology and metabolism* 2001, **280**(5):E788-796.

250. Steiner DF: **The Biosynthesis of Insulin: Genetic, Evolutionary, and Pathophysiologic Aspects**. *Harvey lectures* 1982, **78**:191-228.

251. Steiner DF, Chan SJ, Welsh JM, Kwok SC: **Structure and Evolution of the Insulin Gene**. *Annual review of genetics* 1985, **19**:463-484.

252. Steiner DJ, Kim A, Miller K, Hara M: **Pancreatic Islet Plasticity: Interspecies Comparison of Islet Architecture and Composition**. *Islets* 2010, **2**(3):135-145.

253. Stene LC, Oikarinen S, Hyoty H, Barriga KJ, Norris JM, Klingensmith G, Hutton JC, Erlich HA, Eisenbarth GS, Rewers M: **Enterovirus Infection and Progression from Islet Autoimmunity to Type 1 Diabetes: The Diabetes and Autoimmunity Study in the Young (Daisy)**. *Diabetes* 2010, **59**(12):3174-3180.

254. Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, Edlund H: **The Ffa Receptor Gpr40 Links Hyperinsulinemia, Hepatic Steatosis, and Impaired Glucose Homeostasis in Mouse**. *Cell metabolism* 2005, **1**(4):245-258.

255. Stocker H, Hafen E: **Genetic Control of Cell Size**. *Current opinion in genetics & development* 2000, **10**(5):529-535.

256. Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, White MF: **Structure of the Insulin Receptor Substrate Irs-1 Defines a Unique Signal Transduction Protein**. *Nature* 1991, **352**(6330):73-77.

257. Sun XJ, Wang LM, Zhang Y, Yenush L, Myers MG, Jr., Glasheen E, Lane WS, Pierce JH, White MF: **Role of Irs-2 in Insulin and Cytokine Signalling**. *Nature* 1995, **377**(6545):173-177.

258. Tager HS, Steiner DF, Patzelt C: **Biosynthesis of Insulin and Glucagon**. *Methods in cell biology* 1981, **23**:73-88.

259. Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S *et al*: **Insulin Resistance and Growth Retardation in Mice Lacking Insulin Receptor Substrate-1**. *Nature* 1994, **372**(6502):182-186.

260. Tanaka S, Ito T, Wands JR: **Neoplastic Transformation Induced by Insulin Receptor Substrate-1 Overexpression Requires an Interaction with Both Grb2 and Syp Signaling Molecules**. *The Journal of biological chemistry* 1996, **271**(24):14610-14616.

261. Taniguchi CM, Emanuelli B, Kahn CR: **Critical Nodes in Signalling Pathways: Insights into Insulin Action**. *Nature reviews Molecular cell biology* 2006, **7**(2):85-96.

262. Todd JA: **Etiology of Type 1 Diabetes**. *Immunity* 2010, **32**(4):457-467.

263. Tokui Y, Kozawa J, Yamagata K, Zhang J, Ohmoto H, Tochino Y, Okita K, Iwahashi H, Namba M, Shimomura I *et al*: **Neogenesis and Proliferation of Beta-Cells Induced by Human Betacellulin Gene Transduction Via Retrograde Pancreatic Duct Injection of an Adenovirus Vector**. *Biochemical and biophysical research communications* 2006, **350**(4):987-993.

264. Tortoriello DV, McMinn J, Chua SC: **Dietary-Induced Obesity and Hypothalamic Infertility in Female Dba/2j Mice**. *Endocrinology* 2004, **145**(3):1238-1247.

265. Tortoriello DV, McMinn JE, Chua SC: **Increased Expression of Hypothalamic Leptin Receptor and Adiponectin Accompany Resistance to Dietary-Induced Obesity and Infertility in Female C57bl/6j Mice**. *International journal of obesity* 2007, **31**(3):395-402.

266. Trayhurn P: **Hypoxia and Adipose Tissue Function and Dysfunction in Obesity**. *Physiological reviews* 2013, **93**(1):1-21.

267. Tremblay F, Brule S, Hee Um S, Li Y, Masuda K, Roden M, Sun XJ, Krebs M, Polakiewicz RD, Thomas G *et al*: **Identification of Irs-1 Ser-1101 as a Target of S6k1 in Nutrient- and Obesity-Induced Insulin Resistance**. *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**(35):14056-14061.

268. Tsai CH, Hill M, Asa SL, Brubaker PL, Drucker DJ: **Intestinal Growth-Promoting Properties of Glucagon-Like Peptide-2 in Mice**. *The American journal of physiology* 1997, **273**(1 Pt 1):E77-84.

269. Tschopp O, Yang ZZ, Brodbeck D, Dummler BA, Hemmings-Mieszczak M, Watanabe T, Michaelis T, Frahm J, Hemmings BA: **Essential Role of Protein Kinase B Gamma (Pkb Gamma/Akt3) in Postnatal Brain Development but Not in Glucose Homeostasis**. *Development* 2005, **132**(13):2943-2954.

270. Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furth EE, Polonsky KS, Naji A, Birnbaum MJ: **Regulation of Pancreatic Beta-Cell Growth and Survival by the Serine/Threonine Protein Kinase Akt1/Pkbalpha**. *Nature medicine* 2001, **7**(10):1133-1137.

271. Uchizono Y, Alarcon C, Wicksteed BL, Marsh BJ, Rhodes CJ: **The Balance between Proinsulin Biosynthesis and Insulin Secretion: Where Can Imbalance Lead?** *Diabetes, obesity & metabolism* 2007, **9 Suppl 2**:56-66.

272. Ueki K, Okada T, Hu J, Liew CW, Assmann A, Dahlgren GM, Peters JL, Shackman JG, Zhang M, Artner I *et al*: **Total Insulin and Igf-I Resistance in Pancreatic Beta Cells Causes Overt Diabetes**. *Nature genetics* 2006, **38**(5):583-588.

273. Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J *et al*: **Absence of S6k1 Protects against Age- and Diet-Induced Obesity While Enhancing Insulin Sensitivity**. *Nature* 2004, **431**(7005):200-205.

274. Vaillant CR, Lund PK: **Distribution of Glucagon-Like Peptide I in Canine and Feline Pancreas and Gastrointestinal Tract**. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 1986, **34**(9):1117-1121.

275. van Citters GW, Kabir M, Kim SP, Mittelman SD, Dea MK, Brubaker PL, Bergman RN: **Elevated Glucagon-Like Peptide-1-(7-36)-Amide, but Not Glucose, Associated with Hyperinsulinemic Compensation for Fat Feeding**. *The Journal of clinical endocrinology and metabolism* 2002, **87**(11):5191-5198.

276. Van Schravendijk CF, Foriers A, Van den Brande JL, Pipeleers DG: **Evidence for the Presence of Type I Insulin-Like Growth Factor Receptors on Rat Pancreatic a and B Cells**. *Endocrinology* 1987, **121**(5):1784-1788.

277. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B: **The Emerging Mechanisms of Isoform-Specific Pi3k Signalling**. *Nature reviews Molecular cell biology* 2010, **11**(5):329-341.

278. Verspohl EJ, Schenzle D, Ammon HP: **Properties of the Insulin Receptor of Rat Pancreatic Islet**. *Biochimica et biophysica acta* 1982, **716**(2):258-265.

279. Wakelam MJ, Murphy GJ, Hruby VJ, Houslay MD: **Activation of Two Signal-Transduction Systems in Hepatocytes by Glucagon**. *Nature* 1986, **323**(6083):68-71.

280. Wan M, Easton RM, Gleason CE, Monks BR, Ueki K, Kahn CR, Birnbaum MJ: **Loss of Akt1 in Mice Increases Energy Expenditure and Protects against Diet-Induced Obesity**. *Mol Cell Biol* 2012, **32**(1):96-106.

281. Weigert C, Brodbeck K, Staiger H, Kausch C, Machicao F, Haring HU, Schleicher ED: **Palmitate, but Not Unsaturated Fatty Acids, Induces the Expression of Interleukin-6 in Human Myotubes through Proteasome-Dependent Activation of Nuclear Factor-Kappab**. *The Journal of biological chemistry* 2004, **279**(23):23942-23952.

282. Werner H, Weinstein D, Bentov I: **Similarities and Differences between Insulin and Igf-I: Structures, Receptors, and Signalling Pathways**. *Archives of physiology and biochemistry* 2008, **114**(1):17-22.

283. White MF: **The Insulin Signalling System and the Irs Proteins**. *Diabetologia* 1997, **40 Suppl 2**:S2-17.

284. White MF: **Irs Proteins and the Common Path to Diabetes**. *American journal of physiology Endocrinology and metabolism* 2002, **283**(3):E413-422.

285. White MF: **Regulating Insulin Signaling and Beta-Cell Function through Irs Proteins**. *Canadian journal of physiology and pharmacology* 2006, **84**(7):725-737.

286. Wicksteed B, Brissova M, Yan W, Opland DM, Plank JL, Reinert RB, Dickson LM, Tamarina NA, Philipson LH, Shostak A *et al*: **Conditional Gene Targeting in Mouse Pancreatic Ss-Cells: Analysis of Ectopic Cre Transgene Expression in the Brain**. *Diabetes* 2010, **59**(12):3090-3098.

287. Wiebe PO, Kormish JD, Roper VT, Fujitani Y, Alston NI, Zaret KS, Wright CV, Stein RW, Gannon M: **Ptf1a Binds to and Activates Area Iii, a Highly Conserved Region of the Pdx1 Promoter That Mediates Early Pancreas-Wide Pdx1 Expression**. *Molecular and cellular biology* 2007, **27**(11):4093-4104.

288. Withers DJ, Burks DJ, Towery HH, Altamuro SL, Flint CL, White MF: **Irs-2 Coordinates Igf-1 Receptor-Mediated Beta-Cell Development and Peripheral Insulin Signalling**. *Nature genetics* 1999, **23**(1):32-40.

289. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI *et al*: **Disruption of Irs-2 Causes Type 2 Diabetes in Mice**. *Nature* 1998, **391**(6670):900-904.

290. Woodgett JR: **Recent Advances in the Protein Kinase B Signaling Pathway**. *Current opinion in cell biology* 2005, **17**(2):150-157.

291. Wrede CE, Dickson LM, Lingohr MK, Briaud I, Rhodes CJ: **Protein Kinase B/Akt Prevents Fatty Acid-Induced Apoptosis in Pancreatic Beta-Cells (Ins-1)**. *J Biol Chem* 2002, **277**(51):49676-49684.

292. http://www.uniprot.org

293. Xu G, Marshall CA, Lin TA, Kwon G, Munivenkatappa RB, Hill JR, Lawrence JC, Jr., McDaniel ML: **Insulin Mediates Glucose-Stimulated Phosphorylation of Phas-I by Pancreatic Beta Cells. An Insulin-Receptor Mechanism for Autoregulation of Protein Synthesis by Translation**. *The Journal of biological chemistry* 1998, **273**(8):4485-4491.

294. Xu P, Jacobs AR, Taylor SI: **Interaction of Insulin Receptor Substrate 3 with Insulin Receptor, Insulin Receptor-Related Receptor, Insulin-Like Growth Factor-1 Receptor, and Downstream Signaling Proteins**. *The Journal of biological chemistry* 1999, **274**(21):15262-15270.

295. Yamauchi T, Tobe K, Tamemoto H, Ueki K, Kaburagi Y, Yamamoto-Honda R, Takahashi Y, Yoshizawa F, Aizawa S, Akanuma Y *et al*: **Insulin Signalling and Insulin Actions in the Muscles and Livers of Insulin-Resistant, Insulin Receptor Substrate 1-Deficient Mice**. *Molecular and cellular biology* 1996, **16**(6):3074-3084.

296. Yang Y, Smith DL, Jr., Keating KD, Allison DB, Nagy TR: **Variations in Body Weight, Food Intake, and Body Composition after Long-Term High-Fat Diet Feeding in C57bl/6j Mice**. *Obesity* 2014.

297. Yang ZZ, Tschopp O, Di-Poi N, Bruder E, Baudry A, Dummler B, Wahli W, Hemmings BA: **Dosage-Dependent Effects of Akt1/Protein Kinase Balpha (Pkbalpha) and Akt3/Pkbgamma on Thymus, Skin, and Cardiovascular and Nervous System Development in Mice**. *Molecular and cellular biology* 2005, **25**(23):10407-10418.

298. Yang ZZ, Tschopp O, Hemmings-Mieszczak M, Feng J, Brodbeck D, Perentes E, Hemmings BA: **Protein Kinase B Alpha/Akt1 Regulates Placental Development and Fetal Growth**. *The Journal of biological chemistry* 2003, **278**(34):32124-32131.

299. Ye J: **Role of Insulin in the Pathogenesis of Free Fatty Acid-Induced Insulin Resistance in Skeletal Muscle**. *Endocrine, metabolic & immune disorders drug targets* 2007, **7**(1):65-74.

300. Ye J: **Regulation of Ppargamma Function by Tnf-Alpha**. *Biochemical and biophysical research communications* 2008, **374**(3):405-408.

301. Ye J: **Emerging Role of Adipose Tissue Hypoxia in Obesity and Insulin Resistance**. *International journal of obesity* 2009, **33**(1):54-66.

302. Ye J: **Mechanisms of Insulin Resistance in Obesity**. *Frontiers of medicine* 2013, **7**(1):14-24.

303. Ye J, Gimble JM: **Regulation of Stem Cell Differentiation in Adipose Tissue by Chronic Inflammation**. *Clinical and experimental pharmacology & physiology* 2011, **38**(12):872-878.

304. Yu Z, Jin T: **New Insights into the Role of Camp in the Production and Function of the Incretin Hormone Glucagon-Like Peptide-1 (Glp-1)**. *Cellular signalling* 2010, **22**(1):1-8.

305. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE: **Reversal of Obesity- and Diet-Induced Insulin Resistance with Salicylates or Targeted Disruption of Ikkbeta**. *Science* 2001, **293**(5535):1673-1677.

306. Yun SJ, Tucker DF, Kim EK, Kim MS, Do KH, Ha JM, Lee SY, Yun J, Kim CD, Birnbaum MJ *et al*: **Differential Regulation of Akt/Protein Kinase B Isoforms During Cell Cycle Progression**. *FEBS letters* 2009, **583**(4):685-690.

307. Zanone MM, Favaro E, Camussi G: **From Endothelial to Beta Cells: Insights into Pancreatic Islet Microendothelium**. *Current diabetes reviews* 2008, **4**(1):1-9.

308. Zhang H, Fujitani Y, Wright CV, Gannon M: **Efficient Recombination in Pancreatic Islets by a Tamoxifen-Inducible Cre-Recombinase**. *Genesis* 2005, **42**(3):210-217.

309. Zhang J, Gao Z, Yin J, Quon MJ, Ye J: **S6k Directly Phosphorylates Irs-1 on Ser-270 to Promote Insulin Resistance in Response to Tnf-(Alpha) Signaling through Ikk2**. *The Journal of biological chemistry* 2008, **283**(51):35375-35382.

310. Zhang X, Gan L, Pan H, Guo S, He X, Olson ST, Mesecar A, Adam S, Unterman TG: **Phosphorylation of Serine 256 Suppresses Transactivation by Fkhr (Foxo1) by Multiple Mechanisms. Direct and Indirect Effects on Nuclear/Cytoplasmic Shuttling and DNA Binding**. *The Journal of biological chemistry* 2002, **277**(47):45276-45284.

311. Zhou GL, Tucker DF, Bae SS, Bhatheja K, Birnbaum MJ, Field J: **Opposing Roles for Akt1 and Akt2 in Rac/Pak Signaling and Cell Migration**. *The Journal of biological chemistry* 2006, **281**(47):36443-36453.

312. Zhou L, Chen H, Xu P, Cong LN, Sciacchitano S, Li Y, Graham D, Jacobs AR, Taylor SI, Quon MJ: **Action of Insulin Receptor Substrate-3 (Irs-3) and Irs-4 to Stimulate Translocation of Glut4 in Rat Adipose Cells**. *Molecular endocrinology* 1999, **13**(3):505-514.

313. Zhuravleva E. T, O. and Hemmings, B.A. : **Role of Pkb/Akt in Liver Diseases.** *Signaling Pathways in Liver Diseases (Dufour J-F and Clavien P-A, eds), Springer, Berlin/Heidelberg,* 2010:243-261.

314. Zucker LZT: **Fatty, a New Mutation in the Rat**. *Journal of Heredity* 1961, **52**(6):275-278.

7. Acknowledgements

I would like to thank:

My supervisor Markus Niessen for giving me the opportunity to work on this project and for his valuable support and the numerous scientific discussions.

Giatgen Spinas for his continuous interest in my work, his suggestions and input, and for encouraging and supporting me to present my project at several conferences.

My thesis committee members, Matthias Peter, Ernst Hafen, and Daniel Konrad for valuable guidance and scientific comments.

Oliver Tschopp for kindly providing transgenic mice, for fruitful collaborations and scientific contributions.

Richard Züllig for all his help and expertise, his constant support in designing and performing my experiments, for reading and correcting the thesis and for constantly lifting my mood. Thank you for making my PhD so much more enjoyable.

Heidi Seiler and Dora Schmid for technical support, helpfulness and entertaining conversations.

Present and past members from the C-floor for discussions, scientific advice and the nice work environment. Thank you very much Stefan for the helpful inputs in planning and performing animal experiments and your scientific comments. Thank you Christoph for your various scientific inputs and explanations. Thank you Stefan, Flurin, Fabrizio, Diri, Fausto Heidi and Claudia for amusing and interesting discussion at lunch times.

My friends for the nice times we spent during my studies and my PhD. Your humour always cheers me up and makes everything easier. Special thanks go to Benjamin for your support and amusing times during all of my studies. Thank you for your loyal and enduring friendship.

My parents Monika and Michael, my sisters Jeannine and Christiane, and her family Malu and Renato. Thank you for your confidence, love and support and for encouraging me during all of my studies. Thank you Mama for your constant help and for always cheering me up. Thank you Papa for teaching me to be curious, interested, and open to new things.

Michael for your confidence and love, and for being with me at all times. It’s nice to have you to go trough good and bad times.

8. Curriculum vitae

General information

Name:	Maren Gil Dietrich
Date of birth:	September 19th, 1983
Citizenship:	Swiss
Address:	In der Ey 80, CH-8047 Zurich
Phone:	+41 79 447 87 74
E-Mail:	maren.dietrich@usz.ch

Education

since 2011: **PhD Studies at University Hospital Zurich, Zurich, Switzerland**
Division of Endocrinology, Diabetes and Clinical Nutrition, Dr. Markus Niessen

Member of the PhD program "Systems Biology of Complex Diseases" from Life Science Zurich Graduate School

Supervisors: Dr. Markus Niessen, Prof. Dr. Matthias Peter (ETH Zurich)

08/2010: **Eidg. dipl. Apothekerin ETH, ETH Zurich, Zurich, Switzerland**

2008 - 2010: **Master of pharmaceutical sciences, ETH Zurich, Zurich, Switzerland**
Master Thesis: Regulation of neurogenesis in the mouse olfactory epithelium: First insight in the turnover rate of microvillar cells.

Supervisor: Prof. Dr. Jean-Marc Fritschy, Neuroscience Center Zurich, Zurich, Switzerland

2003 - 2008: **Bachelor of pharmaceutical sciences, ETH Zurich, Zurich, Switzerland**
Project work: Characterisation of translationally regulated mRNAs by phenotypic analysis of deletion strains.

Supervisor: Prof. Dr. Michael Detmar, Institute of pharmaceutical sciences, ETH Zurich, Zurich, Switzerland

2002 - 2003: Biochemistry, ETH Zurich, Zurich, Switzerland

1998 - 2002: Institute Montana, Zugerberg, Switzerland
Matura typus E

Oral & poster presentations

06/2014: 74th Scientific Sessions of ADA (American Diabetes Association), San Francisco, USA (Oral presentation)

11/2013: Annual Congress of SGED (Schweizerische Gesellschaft für Endokrinologie und Diabetologie), Bern, Switzerland (Poster presentation)

10/2013: 49th Annual Meeting of EASD (European Association for the Study of Diabetes), Barcelona, Spain (Poster presentation)

11/2012: 7th Annual Retreat, Competence Center for Systems Physiology and Metabolic Diseases, Ittingen, Switzerland (Oral presentation)

11/2012: Annual Congress of SGED (Schweizerische Gesellschaft für Endokrinologie und Diabetologie), Bern, Switzerland (Poster presentation)

11/2012: 48th Annual Meeting of EASD (European Association for the Study of Diabetes), Berlin, Germany (Poster presentation)

Grants

2014-2016 Amelie Waring Stiftung, Zurich

2014-2015 Forschungskredit der Universität Zürich (UZH), Zurich

Advanced training

since 2011: Member of the human islet transplantation team, University Hospital Zurich, Zurich, Switzerland
Division of Endocrinology, Diabetes and Clinical Nutrition
Supervisor: Prof. Dr. Roger Lehmann

03/2013: Training for persons responsible for directing animal experiments
LTK Module 2, Institut für Labortierkunde, University of Zurich, Zurich, Switzerland

07/2011: Introductory course in laboratory animal science
LTK Module 1, Institut für Labortierkunde, University of Zurich, Zurich, Switzerland

Professional activities

Since 2011: Pharmacy “Adler-Apotheke”, Winterthur, Switzerland
Working as pharmacist (10%)

2009 - 2010: Pharmacy “Helvetiaplatz Apotheke”, Zurich, Switzerland
Clinical traineeship

2007: Carmen Rico, Zurich, Switzerland
Domestic aid

07/2003: Hugo Mendel Stiftung, Zurich, Switzerland
Care of woman suffering from cancer

Additional information

Language Skills: German (mother tongue), English (fluent), French (intermediate), Portuguese (basic knowledge)

Personal Interests: German literature, Theatre

Publications

Schultze SM, **Dietrich MG**, Hynx D, Geier A, Niessen M, Spinas GA, Hemmings BA and Tschopp O. Reduced hepatic lipid content in Pten-haplodeficient mice due to enhanced Akt2/PKBβ activation in skeletal muscle. Liv Int. 2013, 2014

Pfister S*, **Dietrich MG***, Sidler C, Fritschy JM, Knuesel I and Elsaesser R. Characterization and turnover of CD73/IP(3)R3-positive microvillar cells in the adult mouse olfactory. Chem. Senses vol. 37 pp. 859-68, 2012
*Authors contributed equally to the work

Dietrich MG, Zuellig RA, Spinas GA, Lehmann R, Tschopp O and Niessen M. Specific and redundant roles of PKBα/AKT1 and PKBβ/AKT2 in human pancreatic islets, 2014, manuscript submitted to Archives of Physiology and Biochemistry

Published abstracts

Dietrich MG, Zuellig RA, Spinas GA, et al. PKBα Regulates Pancreatic β-Cell Mass. 74th Scientific Sessions of ADA (American Diabetes Association), San Francisco, USA Diabetes, vol. 63, supp. 1 pp. 2899-2900, abstract 41, June 2014.

Dietrich MG, Zuellig RA, Hemmings BA, et al. PKBalpha but not PKBbeta can protect human islets from IL-1beta-induced apoptosis. 49th Annual Meeting of the European Association of the study of diabetes (EASD), Barcelona, Spain. Diabetologia, vol. 56, supp. 1 pp. 220-220, abstract 531, September 2013.

Dietrich MG, Zuellig RA, Spinas GA, et al. Pkb/akt isoforms and the regulation of pancreatic beta cell mass and function. 48th Annual Meeting of the European Association of the study of diabetes (EASD), Berlin, Germany. Diabetologia, vol. 55, supp. 1 pp. 166-167, abstract 388, October 2012.

9. Appendix

9.1 Reduced hepatic lipid content in *Pten*-haplodeficient mice because of enhanced AKT2/PKBβ activation in skeletal muscle

Simon M. Schultze [1,2]; **Maren G. Dietrich**[1,5]; Debby Hynx[2]; Andreas Geier[3,4]; Markus Niessen[1,5]; Giatgen A. Spinas[1,5]; Brian A. Hemmings[2]; Oliver Tschopp[1,5],

[1] Department of Endocrinology, Diabetes & Clinical Nutrition, University Hospital of Zurich, Switzerland

[2] Friedrich Miescher Institute (FMI) for Biomedical Research, Basel, Switzerland

[3] Division of Hepatology, Department of Medicine II, University Hospital Wuerzburg, Wuerzburg, Germany

[4] Department of Gastroenterology and Hepatology, University Hospital Zurich, Zurich, Switzerland

[5] Competence Center for Systems Physiology and Metabolic Diseases, Zurich, Switzerland

Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

ORIGINAL ARTICLE

Reduced hepatic lipid content in *Pten*-haplodeficient mice because of enhanced AKT2/PKBβ activation in skeletal muscle

Simon M. Schultze[1,2], Maren Dietrich[1], Debby Hynx[2], Andreas Geier[3,4], Markus Niessen[1,5], Giatgen A. Spinas[1,5], Brian A. Hemmings[2] and Oliver Tschopp[1,5]

1 Department of Endocrinology, Diabetes & Clinical Nutrition, University Hospital Zurich, Zurich, Switzerland
2 Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland
3 Division of Hepatology, Department of Medicine II, University Hospital Wuerzburg, Wuerzburg, Germany
4 Department of Gastroenterology and Hepatology, University Hospital Zurich, Zurich, Switzerland
5 Competence Centre for Systems Physiology and Metabolic Diseases, Swiss Federal Institute of Technology (ETH) Zurich, Zurich, Switzerland

Keywords
liver – metabolism – NAFLD

Correspondence
Oliver Tschopp, MD
University Hospital Zurich
Department of Endocrinology, Diabetes and Clinical Nutrition
Raemistrasse 100; 8091 Zurich, Switzerland
Tel: +41 (0) 44 255 3620
Fax: +41 (0) 44 255 4447
e-mail: oliver.tschopp@usz.ch

Received 21 November 2013
Accepted 14 May 2014

DOI:10.1111/liv.12600

Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is a major health problem and occurs frequently in the context of metabolic syndrome and type 2 diabetes mellitus. Hepatocyte-specific *Pten*-deficiency in mice was shown previously to result in hepatic steatosis due to hyperactivated AKT2. However, the role of peripheral insulin-sensitive tissues on PTEN- and AKT2-dependent accumulation of hepatic lipids has not been addressed. *Methods:* Effects of systemically perturbed PTEN/AKT2 signalling on hepatic lipid content were studied in *Pten*-haplodeficient ($Pten^{+/-}/Akt2^{+/+}$) mice and *Pten*-haplodeficient mice lacking *Akt2* ($Pten^{+/-}/Akt2^{-/-}$). The liver and skeletal muscle were characterized by histology and/or analysis of insulin signalling. To assess the effects of AKT2 activity in skeletal muscle on hepatic lipid content, AKT2 mutants were expressed in skeletal muscle of $Pten^{+/+}/Akt2^{+/+}$ and $Pten^{+/-}/Akt2^{+/+}$ mice using adeno-associated virus 8. *Results:* $Pten^{+/-}/Akt2^{+/+}$ mice were found to have a more than 2-fold reduction in hepatic lipid content, at a level similar to that observed in $Pten^{+/-}/Akt2^{-/-}$ mice. Insulin signalling in the livers of $Pten^{+/-}/Akt2^{+/+}$ mice was enhanced, indicating that extrahepatic factors prevent lipid accumulation. The skeletal muscle of $Pten^{+/-}/Akt2^{+/+}$ mice also showed enhanced insulin signalling. Skeletal muscle-specific expression of constitutively active AKT2 reduced hepatic lipid content in $Pten^{+/+}/Akt2^{+/+}$ mice, and dominant negative AKT2 led to an increase in accumulation of hepatic lipids in both $Pten^{+/+}/Akt2^{+/+}$ and $Pten^{+/-}/Akt2^{+/+}$ mice. *Conclusion:* Our results demonstrate that AKT2 activity in skeletal muscle critically affects lipid accumulation in the livers of $Pten^{+/+}/Akt2^{+/+}$ and $Pten^{+/-}/Akt2^{+/+}$ mice, and emphasize the role of skeletal muscle in the pathology of NAFLD.

Non-alcoholic fatty liver disease (NAFLD) is a major complication in patients with metabolic syndrome and type 2 diabetes mellitus (T2D) and affects approximately one-third of adults and about 10% of children in developed countries (1–3). NAFLD is characterized by the accumulation of predominantly macrovesicular lipid droplets in the cytoplasm of hepatocytes (4). A substantial number of patients with NAFLD develop necro-inflammatory changes in the liver (non-alcoholic steatohepatitis, NASH), which may lead to cirrhosis and eventually to hepatocellular carcinoma and hepatic failure (4, 5). Although the association of NAFLD with insulin resistance and T2D is well known, the molecular mechanisms have not been fully elucidated. It is considered that insulin resistance of skeletal muscle and/or selectively impaired hepatic insulin signalling drive ectopic lipid accumulation in the liver (6–8).

Insulin is indispensable for the regulation of systemic metabolism. It stimulates cellular glucose uptake and induces anabolic processes, largely mediated by AKT (9). The AKT family of serine/threonine kinases consist of three isoforms (AKT1/PKBα, AKT2/PKBβ, AKT3/PKBγ) and AKT2 is considered to be the major isoform downstream of the insulin receptor (10, 11). Mice lacking *Akt2* develop insulin resistance and a T2D-like syndrome because of severe hepatic and skeletal muscle insulin resistance (12, 13).

PTEN antagonizes activation of AKT and, among diverse other growth factors, negatively regulates insulin action (9). While homozygous deletion of *Pten* in mice causes embryonic lethality, *Pten*-haplodeficient (*Pten*$^{+/-}$) mice are viable (14). Such mice show improved glucose tolerance and insulin sensitivity, but with age *Pten*$^{+/-}$ mice frequently develop tumours in liver, colon and thyroid glands (14, 15). Similarly, loss of function mutations of *Pten* in patients are associated with Cowden's disease, a rare cancer-predisposition syndrome (16). In addition to the development of tumours in various organs, such patients show enhanced insulin sensitivity of the liver and skeletal muscle (17).

Recently, reduced expression of PTEN and concomitant hyperactivation of AKT were found in liver biopsies from patients with NAFLD (18). Indeed, mice with hepatocyte-specific deletion of *Pten* show spontaneous accumulation of hepatic lipids starting at 10 weeks of age and severe hepatic steatosis and hepatocellular carcinoma develop in an age-dependent manner (19, 20). Overall, the reported phenotype is similar to the pathology of human NAFLD and its forms of progression. It was shown that accumulation of hepatic lipids in these mice is driven by hyperactivated AKT2 that upregulates lipogenic genes such as FAS and ACC, most likely in a hepatocyte-autonomous manner (19–21). In addition, mice with hepatocyte-specific deletion of PTEN show improved glucose tolerance, which is likely a result of AKT2-mediated downregulation of G6Pase and PEPCK and inhibition of hepatic gluconeogenesis (19–21). It was suggested that hepatic steatosis in insulin-resistant patients is promoted by the selective failure of insulin to inhibit hepatic gluconeogenesis, but not *de novo* lipogenesis (6). Although it was shown that hepatic insulin signalling diverge downstream of AKT, the molecular mechanism and its significance in insulin resistant patients is not fully understood (22).

However, the metabolic state of the liver also depends on systemic metabolism, which is regulated by multiple insulin-sensitive tissues. Donelly and colleagues showed that in obese patients approximately 60% of lipids in the liver are derived from a serum pool of non-esterfied fatty acids (NEFA), 25% originate from *de novo* lipogenesis in the liver and 15% are derived directly from dietary intake (23). Furthermore, it was shown that hepatic *de novo* lipogenesis is inversely correlated to skeletal muscle insulin sensitivity in different subsets of patients (7, 24, 25). The role of peripheral insulin-sensitive tissues on PTEN/AKT2-dependent accumulation of hepatic lipids has not yet been addressed. In the present study, we analysed mice with whole-body *Pten*-haplodeficiency (*Pten*$^{+/-}$/*Akt2*$^{+/+}$) and found that PTEN expression in extrahepatic tissues affects accumulation of lipids in PTEN-deficient livers. *Pten*-haplodeficient mice lacking *Akt2* (*Pten*$^{+/-}$/*Akt2*$^{-/-}$) were used to dissect the role of AKT2 in this mouse model.

In contrast to hepatic steatosis reported in mice with hepatocyte-specific deletion of *Pten*, we show here that hepatic lipid content of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice is reduced by 60% compared to control mice and at a level similar to that observed in *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice. In contrast to the reduced lipid content, *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice showed enhanced insulin signalling in the liver, consistent with the notion that extrahepatic factors prevent lipid accumulation in the livers of these mice. As hepatic lipid content in humans correlates with insulin action in skeletal muscle, we examined if and how regulation and function of AKT2 in skeletal muscle contribute to reduced hepatic lipid content in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice (7, 24, 25). Indeed, insulin response of skeletal muscle in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice was enhanced as evidenced by increased glycogen content and activation of AKT2. Significantly, skeletal muscle-specific expression of constitutively active AKT2 reduced hepatic lipid accumulation in *Pten*$^{+/+}$/*Akt2*$^{+/+}$ mice and dominant negative AKT2 increased hepatic lipid content in both *Pten*$^{+/+}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice.

Material and methods

Mice

All animal experiments were performed in accordance with Swiss Federal Animal Regulations and approved by the Veterinary Offices of Zurich and Basel, Switzerland. Mice with whole-body targeted deletion of *Pten* and *Akt2* were described previously and were in a C57BL/6 background after at least six backcrosses (13, 14). *Pten*$^{+/+}$/*Akt2*$^{+/+}$, *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice were obtained by crossing *Pten*$^{+/-}$/*Akt2*$^{+/-}$ mice. *Akt2*$^{+/+}$ and *Akt2*$^{-/-}$ mice were obtained by crossing *Akt2*$^{+/-}$ mice. The experimental mice were 20- to 22-week-old males. Mice were fasted by removing food for 8 h during the dark cycle. Fasted-refed mice were refed for 2 h after 8 h of fasting. Insulin stimulation was performed in fasted mice by injection of human recombinant insulin at 1 U/kg of body weight (Novo Nordisk, Kuesnacht, Switzerland) via the inferior vena cava; samples were collected after 20 min. Mice are alive but terminally anesthetized during insulin stimulation. Mice were housed in groups with a 12-h dark-light cycle and free access to food and water, unless otherwise indicated.

Vector production and administration

Adeno-associated virus 8 (AAV8) vectors were generated by triple plasmid transfection of HEK293T cells using jetPEI (Polyplus, Illkirch, France). AAV transplasmid (serotype 8) and helper plasmid were obtained from Penn Vector Core, Philadelphia, PA. myr-AKT2 and AKT2^{K180A} were cloned as described previously and cloned into AAV expression vector containing a CMV promoter and a 2A-GFP reporter gene (26, 27). Viral particles were purified using a discontinuous iodixanol gradient as previously described (28). Titers

were determined by quantitative real-time PCR. 2×10^{11} genome copies of AAV8 viral particles were administered to 4-day-old mice by intraperitoneal injection to achieve skeletal muscle-specific transgene expression as previously described (29). Mice used for subsequent metabolic analyses were 18- to 20-week old.

Analysis of metabolic parameters in blood and tissues

Glucose levels were measured in tail vein blood using a glucose meter Freestyle (Disetronic, Burgdorf, Switzerland). Glucose tolerance tests were performed with fasted mice by intraperitoneal injection of 2 g D-(+)-glucose anhydrous/kg of body weight (Fluka, Buchs, Switzerland) and glucose levels measured at indicated time points. Triglyceride contents of skeletal muscle and liver and glycogen contents of skeletal muscle were determined as described previously (30).

Histology and quantitative analysis

Haematoxylin and eosin (H&E) and Oil Red O (Sigma-Aldrich, Saint Louis, MO, USA) staining were performed according to standard protocols on paraffin and frozen sections respectively. For fluorescent staining of lipids, frozen sections were fixed in 10% formaldehyde, incubated with 1 μg/ml BODIPY493/503 (Invitrogen, Carlsbad, CA, USA) in 150 mM NaCl for 20 min at room temperature, and counterstained with 4′,6- Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich). BOPIPY493/503-stained areas relative to total tissue areas were quantified using Imaris software (Bitplane, Zurich, Switzerland). GFP staining was performed using Ventana DiscoveryXT (Roche Diagnostics, Mannheim, Germany) with a customized procedure for fluorescent staining. Slides were pretreated with mild CC1, incubated with anti-GFP antibody (Invitrogen) for 1 h at 37°C, incubated with goat antirabbit conjugated with Alexa fluor 647 (Invitrogen) for 32 min at 37°C and counterstained manually with DAPI.

Western blot analysis

Western blot analysis was performed using standard protocols (GE Healthcare, Buckinghamshire, UK). Images were captured on film or by BioSpectrum Imaging System (UVP, Cambridge, UK). Signal intensities were quantified by photodensitometry after background subtraction relative to β-Actin and normalized to fasted *Pten*$^{+/+}$/*Akt2*$^{+/+}$ mice. Antibodies against the following proteins were used: PTEN (Nicholas K. Tonks, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA), PTEN, pan-AKT, AKT1, AKT2, p-AKT S473, p-AKT T308, GSK3α/β, p-GSK3β, FoxO1, p-FoxO1 (Cell Signaling, Beverly, MA, USA) and β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Quantitative real-time PCR

Total RNA was isolated from tissues using TRIZOL (Invitrogen) following the manufacturer's instructions. cDNA synthesis was performed with M-MuLV reverse transcriptase (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's instructions. Quantitative real-time PCR reactions were performed using SYBR Green (Invitrogen) on ABI Prism 7000 or StepOnePlus Real-Time PCR System (Applied Biosystems, Rotkreuz, Switzerland). Primer sequences were obtained from PrimerBank (31). The primers used and the corresponding PrimerBank ID were *Acc* (ID: 14211284a1), *Fas* (ID: 30911099a2), *G6Pase* (ID: 31982353a1), *Gck* (ID: 31982798a1), *Pepck* (ID: 7110683a1), *Pgc1a* (ID: 238018130b1), *Ppara* (ID: 31543500a1), *Pparg* (ID: 6755138a2) and *Srebp-1c* (ID: 14161491a1).

Statistical analysis

All data are presented as means ± standard deviation (SD). Data were subjected to Student's *t*-test for statistical significance ($*P < 0.05$; $**P < 0.01$). The numbers of independent biological samples per group used for each analysis are indicated accordingly.

Results

Improved glucose homoeostasis in Pten$^{+/-}$/Akt2$^{+/+}$ mice depends on AKT2

In order to validate our mouse model we performed Western blot analysis to determine levels of PTEN, AKT2 and AKT1 in liver, skeletal muscle and adipose tissue of *Pten*$^{+/+}$/*Akt2*$^{+/+}$, *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice (Fig. 1A, B, C). As expected, *Pten* mutants showed significantly reduced PTEN protein level. AKT2 was undetectable in mice deficient for *Akt2* and AKT1 expression was unchanged. The level of total AKT as determined by pan-AKT antibody is reduced by 67% in liver, 65% in skeletal muscle and 60% in adipose tissue of *Pten*$^{+/-}$/*Akt2*$^{-/-}$ compared to *Pten*$^{+/+}$/*Akt2*$^{+/+}$ mice indicating that AKT2 is the major isoform in these tissues (Fig. 1A, B, C). This observation is in line with previous studies analysing the tissue-specific expression pattern of AKT isoforms and the notion that AKT2 is the major isoform in context of metabolic control (9, 10, 13, 32).

As described previously, aged *Pten*$^{+/-}$ mice frequently develop tumours in liver, colon and thyroid glands (14, 15). To exclude disturbance of metabolic control by tumour burden we analysed 20- to 22-week-old male mice in this study. No substantial tumour development was observed in these mice. Histological analysis did not reveal pancreatic tumours in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice. There were no changes in the islet morphology of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice, but islet area was

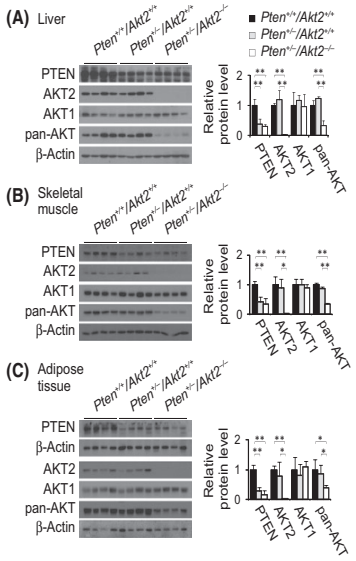


Fig. 1. Validation of gene targeting in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ *and Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice (A, B, C) Protein levels of PTEN, AKT2, AKT1 and pan-AKT in liver (a), skeletal muscle (B) and adipose tissue (C) analysed by Western blotting. Two β-Actin loading controls are shown in panel C as the amount of loaded protein is different. Densitometric quantification is shown. $n = 4$/group. Data are expressed as means ± SD; $*P < 0.05$; $**P < 0.01$.

increased in *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice (data not shown). *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice had body weights similar to controls and perigonadal fat pad weights did not show significant changes (Fig. 2A and data not shown).

Pten$^{+/-}$ mice were previously described as slightly hypoglycaemic with improved glucose tolerance, whereas *Akt2*$^{-/-}$ mice as hyperglycaemic and glucose intolerant (12, 13, 15, 33). Fasting blood glucose concentrations of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice were similar to controls (Fig. 2B). However, when randomly fed or refed after fasting, *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice showed a reduction and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice an increase in blood glucose concentrations (Fig. 2C, D). We performed glucose tolerance tests by intraperitoneal injection of glucose to further assess glycaemic control. The glucose tolerance of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice was improved significantly compared to *Pten*$^{+/+}$*/Akt2*$^{+/+}$ control mice (area under the curve −22.2% ± 18.8%; $P < 0.05$) (Fig. 2E). Interestingly, glucose tolerance of *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice was similar to control mice, indicating compensation of acute glucose challenges (Fig. 2E). In line with previous studies, we found that *Akt2*$^{-/-}$ mice have reduced body weights, increased random fed, but not fasted, blood glucose concentration and significantly impaired glucose tolerance (Fig. 2A, B, C, E) (12, 13).

These data verify the efficacy of gene targeting in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice and also suggest that the reduced blood glucose concentration and improved glucose tolerance of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice partially depend on AKT2.

Hepatic lipid content is reduced in both Pten$^{+/-}$/Akt2$^{+/+}$ and Pten$^{+/-}$/Akt2$^{-/-}$ mice

It was previously reported that mice with a hepatocyte-specific *Pten*-deficiency spontaneously develop hepatic steatosis (19, 20, 34). We analysed the livers of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice to examine the effects of systemically perturbed PTEN/AKT2 signalling on hepatic lipid content.

In contrast to the hepatomegaly reported in mice with hepatocyte-specific *Pten*-deficiency, *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice displayed only minor changes in liver weights (Fig. 3A) (19, 20). To examine the accumulation of hepatic lipids liver sections were stained with H&E, Oil Red O and BODIPY493/503. Hepatic steatosis was not observed in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice but rather lower amounts of lipids were found compared to *Pten*$^{+/+}$*/Akt2*$^{+/+}$ controls (Fig. 3B, C, D). Remarkably, quantification of BODIPY493/503-stained area and measurements of hepatic triglycerides confirmed that hepatic lipid content in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice is reduced by more than 60% and 57%, respectively (Fig. 3E, F). *Akt2*$^{-/-}$ mice show a slightly increased liver to body weight ratio and hepatic triglyceride content is reduced by 52% (Fig. 3A, F).

The observation that *Pten*$^{+/-}$*/Akt2*$^{-/-}$ and *Akt2*$^{-/-}$ mice have reduced hepatic lipid content is in line with previous reports showing that AKT2 in hepatocytes is required for accumulation of hepatic lipids (21, 22). In contrast to hepatic steatosis reported in mice with hepatocyte-specific *Pten*-deficiency, *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice display reduced hepatic lipid content.

Enhanced insulin signalling in the liver of Pten$^{+/-}$/Akt2$^{+/+}$ mice is partially dependent on AKT2

We analysed activation of AKT and AKT-dependent signalling in liver extracts of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice to test if this correlates with reduced hepatic lipid content observed in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ and *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice.

Fasted *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice displayed a more than 4.5-fold increase in phosphorylation of AKT at S473 (p-AKT S473) and T308 (Fig. 4A, left blots). Fasted *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice showed no change in p-AKT S473 but p-AKT T308 was elevated 3-fold (Fig. 4A, left blots). Insulin-stimulated mice were analysed to assess insulin-dependent activation of AKT. p-AKT S473 but not p-AKT T308 was 32% higher in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice compared to controls after insulin stimulation (Fig. 4A, right blots). The insulin-stimulated increase in p-AKT S473 and T308 in *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice was reduced by 72% and 41% compared to control mice, respectively (Fig. 4A, right blots). Analysis of the phosphorylation of AKT targets GSK3β and FoxO1 in these mice revealed a

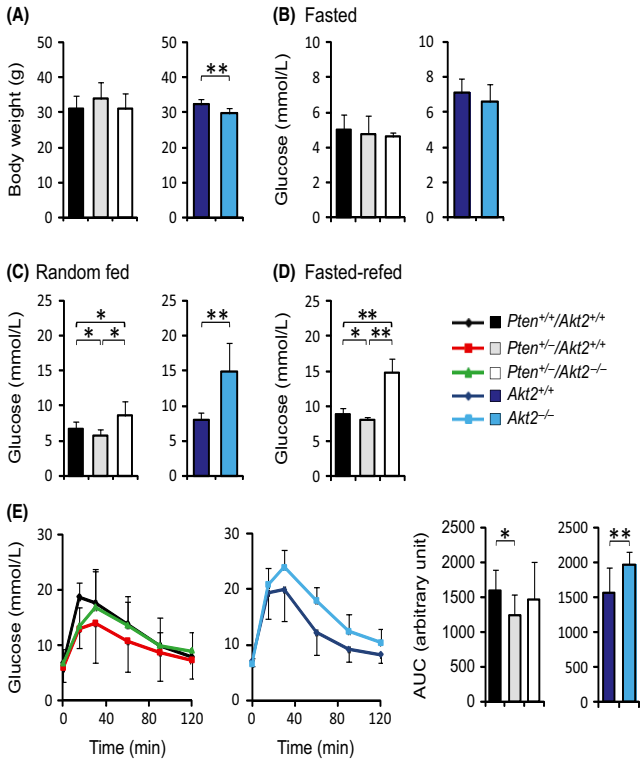


Fig. 2. Improved glycaemic control in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice is dependent on AKT2 (A) Body weights. *n* = 6–8/group. (B, C, D) Blood glucose concentrations from fasted (B), random fed (C) and fasted-refed (D) mice. *n* = 5–11/group. (E) Blood glucose concentrations in fasted mice after intraperitoneal administration of glucose were measured at the indicated time points; respective AUCs are shown. *n* = 7–11/group. AUC, area under the curve; data are expressed as means ± SD; **P* < 0.05; ***P* < 0.01.

high degree of complexity. p-GSK3β was similarly increased in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice during fasting compared to controls, suggesting that AKT2 is not required (Fig. 4B, left blots). However, insulin-stimulated p-GSK3β was increased only in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ but not in *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice, indicating requirement for AKT2 (Fig. 4B, right blots). p-FoxO1 remained unchanged in fasted mice but was increased compared to controls after insulin stimulation in both *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice (Fig. 4B, left and right blots). Fasted *Akt2*$^{-/-}$ mice did not show changes in phosphorylation of AKT at S473 and T308, GSK3β and FoxO1 in the liver (Fig. 4C, D).

To further characterize hepatic insulin signalling, the expression levels of gluconeogenic and lipogenic genes were analysed. While FoxO1 phosphorylation did not change during fasting, expression of *Pepck* and *G6Pase* declined by more than 40% in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice (Fig. 5A). AKT2 was previously shown to upregulate expression of lipogenic genes in mice with hepatocyte-specific *Pten*-deficiency and *leptin*-deficient mice, but not in mice fed with normal chow or a high-fat diet enriched in simple carbohydrates (21, 22). Notably, the lipogenic genes *Srebp1-c* and its targets *Fas* and *Acc* were upregulated in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice (Fig. 5B). In *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice, expression of *Srebp1-c*, *Fas* and *Acc* was high but *Pparg* was downregulated compared to control mice (Fig. 5B). *Ppara* and *Pgc1a* may reduce hepatic lipid content by stimulating gluconeogenesis and β-oxidation (35). There was no difference in the expression of *Ppara* or *Pgc1a* in fasted *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ compared to control mice (Fig. 5C).

These data show that hepatic lipid content does not correlate with activation of AKT2 in the liver in *Pten*-deficient mice. *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice have reduced hepatic lipid content despite upregulated lipogenic genes. This is in line with previous reports showing that AKT2 in hepatocytes is required for accumulation of lipids even if lipogenic genes were upregulated (22). *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice have, however, reduced hepatic lipid content in the presence of enhanced AKT2 activation. These observations suggest that extrahepatic processes in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice can reduce hepatic lipid content in the presence of hyperactivated AKT2 in the liver.

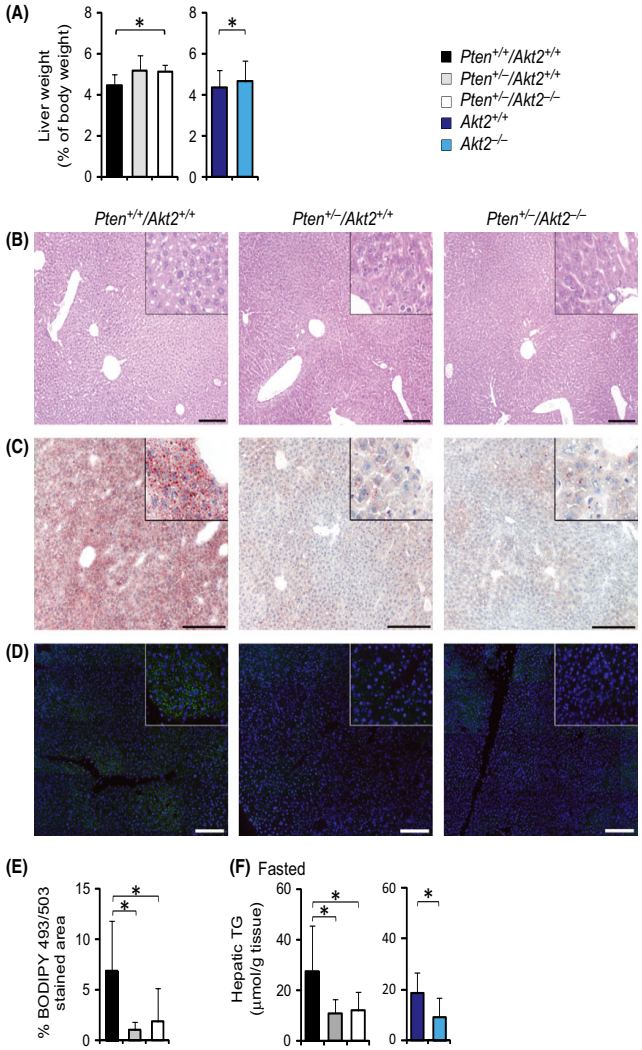


Fig. 3. Reduced hepatic lipid content in *Pten*$^{+/-}$/*Akt2*$^{+/+}$, *Pten*$^{+/-}$/*Akt2*$^{-/-}$ and *Akt2*$^{-/-}$ mice (A) Liver to body weight ratios. (B, C, D) Representative images of liver sections from fasted mice stained with H&E (B), stained for lipids with Oil Red O (C), and stained for lipids and DNA with BODIPY493/503 (green) and DAPI (blue), respectively (D). Image inlays show part of the depicted area at higher magnification. (E) Relative BODIPY493/503-stained area. (F) Hepatic triglyceride content in fasted mice. TG, triglycerides; scale bar = 200 µm; *n* = 6–12/group; data are expressed as means ± SD; **P* < 0.05.

AKT2 is required for increased glycogen and enhanced AKT signalling in skeletal muscle of Pten$^{+/-}$/Akt2$^{+/+}$ mice upon insulin stimulation

It was previously shown that *Pten*$^{+/-}$ mice have increased insulin-induced skeletal muscle glucose uptake (15). Improving skeletal muscle glucose uptake was found to reduce hepatic lipid content in humans (7, 25). Therefore, we examined whether skeletal muscle insulin response contributes to reduced hepatic lipid content in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice.

Glycogen content in skeletal muscle of fasted *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice was similar to controls (Fig. 6A). However, glycogen content was increased in skeletal muscle of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice upon insulin stimulation, but was comparable to controls in *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice (Fig. 6B). Although glycogen content of *Pten*$^{+/-}$/*Akt2*$^{-/-}$ skeletal muscle is not statistical significantly changed compared to *Pten*$^{+/-}$/*Akt2*$^{+/+}$ skeletal there is clear trend towards reduced glycogen content. Mice with enhanced skeletal muscle insulin responsiveness were shown to have increased triglyceride content in

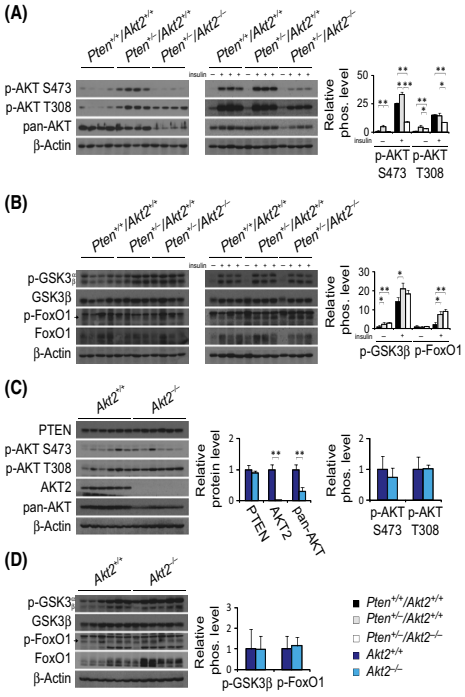


Fig. 4. Enhanced AKT activation in the liver of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice is partially dependent on AKT2 (A) Phosphorylation of AKT at S473 and T308 and pan-AKT protein levels in the liver of fasted and insulin-stimulated mice analysed by Western blotting. Densitometric quantification is shown. n = 3–4/group. (B) Phosphorylation and protein levels of GSK3β and FoxO1 in the liver of fasted and insulin-stimulated mice analysed by Western blotting. Densitometric quantification is shown. n = 3–4/group. (C) Protein levels of PTEN, AKT2 and pan-AKT and phosphorylation of AKT at S473 and T308 in the liver of fasted mice analysed by Western blotting. Densitometric quantification is shown. n = 4/group. (D) Phosphorylation and protein levels of GSK3β and FoxO1 in the liver of fasted mice analysed by Western blotting. Densitometric quantification is shown. n = 4/group. Data are expressed as means ± SD; *P < 0.05; **P < 0.01.

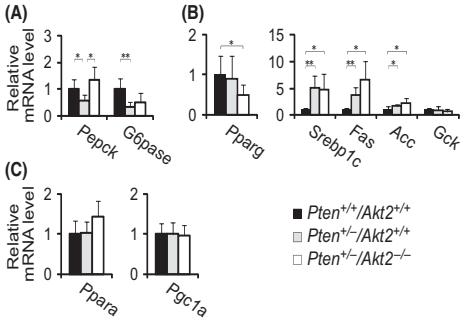


Fig. 5. Expression of gluconeogenic and lipogenic genes in the liver of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice partially depends on AKT2 (A, B, C) Relative mRNA levels of gluconeogenic genes (A), lipogenic genes (B) and genes regulating gluconeogenesis and β-oxidation (C) in the liver of fasted mice analysed by quantitative real-time PCR. n = 6/group. Data are expressed as means ± SD; *P < 0.05; **P < 0.01.

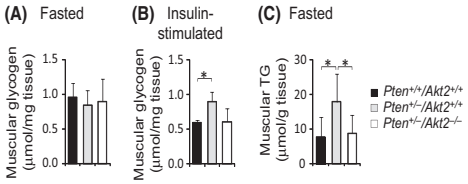


Fig. 6. Increased glycogen and triglyceride content in skeletal muscle of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice. (A, B) Glycogen content in skeletal muscle of fasted (A) and insulin-stimulated (B) mice. fasted n = 6–8/group; insulin-stimulated n = 3/group. (C) Triglyceride content in skeletal muscle of fasted mice. n = 6–8/group. TG, triglycerides; data are expressed as means ± SD; *P < 0.05

skeletal muscle (36). Triglyceride content was increased in skeletal muscle of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ but not *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice (Fig. 6C). In the context of previous studies showing that AKT2 mediates skeletal muscle insulin responsiveness and glucose uptake, these results indicate that AKT2 is involved in enhanced insulin response in skeletal muscle of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice (12, 37).

Next we analysed basal and insulin-dependent activation of AKT and downstream targets in skeletal muscle by Western blotting. AKT phosphorylation in fasted *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice was similar to control mice but, *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice displayed a reduction in p-AKT S473 by more than 70% (Fig. 7A, left blots). There were only minor differences in the phosphorylation of GSK3β and FoxO1 in fasted *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice (Fig. 7B, left blots). In skeletal muscle of insulin-stimulated *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice, p-AKT S473 but not p-AKT T308 was increased by 28% and p-GSK3β and p-FoxO1 were increased by 43% and 61% compared to insulin-stimulated control mice, respectively (Fig. 7A, B, right blots). In *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice, insulin-induced increase in p-AKT S473 and p-AKT T308 was abrogated (Fig. 7A, right blots). Upon insulin stimulation p-GSK3β was increased by 32% and p-FoxO1 was increased by 23% in *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice compared to insulin-stimulated *Pten*$^{+/+}$/*Akt2*$^{+/+}$ mice, which is not as strong as in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice (Fig. 7B, right blots). Fasted *Akt2*$^{-/-}$ mice have reduced p-AKT S473 level, but p-AKT T308, p-GSK3β and p-FoxO1 are not changed (Fig. 7C, D).

These data reveal that the vast majority of insulin-induced phosphorylation of AKT at S473 and T308 in skeletal muscle depends on AKT2. The increase in glycogen and triglyceride content observed in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice is diminished in *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice. In addition the insulin-induced phosphorylation of GSK3β and FoxO1 in *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice is not as strong as in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice. These findings in combination with previous reports on the function of AKT2 in skeletal muscle suggest that the changes observed in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ skeletal muscle are mediated by AKT2.

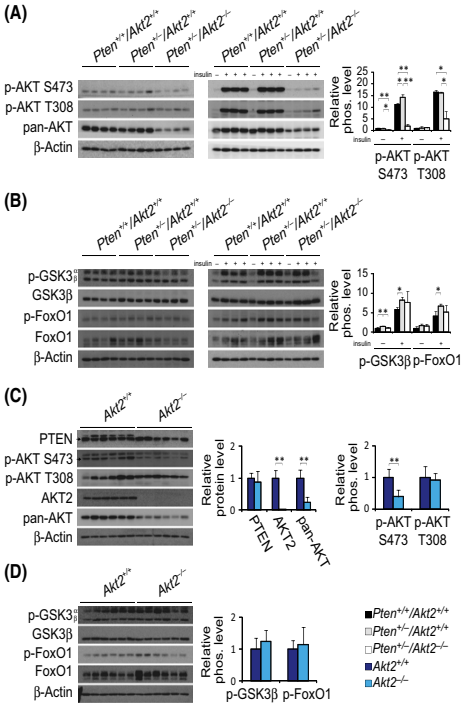


Fig. 7. AKT activation is enhanced in skeletal muscle of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice. (A) Western blot showing phosphorylation of AKT at S473 and T308 and pan-AKT protein levels in skeletal muscle of fasted and insulin-stimulated mice. Densitometric quantification is shown. n = 3–4/group. (B) Phosphorylation and protein levels of GSK3β and FoxO1 in skeletal muscle of fasted and insulin-stimulated mice. Densitometric quantification is shown. n = 3–4/group. (C) Protein levels of PTEN, AKT2 and pan-AKT and phosphorylation of AKT at S473 and T308 in skeletal muscle of fasted mice analysed by Western blotting. Densitometric quantification is shown. n = 4/group. (D) Phosphorylation and protein levels of GSK3β and FoxO1 in skeletal muscle of fasted mice analysed by Western blotting. Densitometric quantification is shown. n = 4/group. Data are expressed as means ± SD; *P < 0.05; **P < 0.01.

Modulation of AKT2 in skeletal muscle affects hepatic lipid content

Skeletal muscle insulin responsiveness is largely mediated by AKT2 and hepatic lipid content was found to be inversely correlated to skeletal muscle insulin sensitivity (7, 10, 12, 24, 25, 37, 38). Since enhanced activation of AKT2 was observed in skeletal muscle of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice, we analysed if modulation of AKT2 in skeletal muscle affects hepatic lipid content in both *Pten*$^{+/+}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice.

Previously, it was shown that injection of adeno-associated virus 8 (AAV8) into neonatal mice leads to robust transgene expression in skeletal muscle of adult mice with minimal expression in non-muscle tissues because of selective retention of the vector DNA (29). We adopted this method to determine if AKT2 in skeletal muscle can affect the accumulation of hepatic lipids. AAV8 vectors encoding constitutively active AKT2 (myr-AKT2) and dominant negative AKT2 (AKT2^{K180A}) with a GFP reporter were constructed. Neonatal *Pten*$^{+/+}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice were injected with our different AAV8 vectors and designated as *Pten*$^{+/+}$/2A.GFP, *Pten*$^{+/+}$/myr-AKT2.2A.GFP, *Pten*$^{+/+}$/AKT2^{K180A}.2A.GFP, *Pten*$^{+/-}$/2A.GFP and *Pten*$^{+/-}$/AKT2^{K180A}.2A.GFP. The specificity of transgene expression was validated by GFP staining of skeletal muscle and liver sections from 18- to 20-week-old mice (Fig. 8). Western blot analysis shows a prominent increase in p-AKT S473, p-AKT T308 and p-GSK3β in skeletal muscle of fasted *Pten*$^{+/+}$/myr-AKT2.2A.GFP mice (Fig. 9A, B). Fasted *Pten*$^{+/-}$/AKT2^{K180A}.2A.GFP show reduced level of p-GSK3β compared to *Pten*$^{+/-}$/2A.GFP control mice (Fig. 9B).

Adult mice expressing AKT2 mutants in skeletal muscle had body weights and fasted blood glucose concentrations similar to GFP-expressing control mice (data not shown). Glucose tolerance tests were performed to further assess glycaemic control. *Pten*$^{+/+}$/myr-AKT2.2A.GFP and *Pten*$^{+/-}$/AKT2^{K180A}.2A.GFP showed glucose tolerance similar to the respective control mice. However, glucose tolerance of *Pten*$^{+/+}$/AKT2^{K180A}.2A.GFP mice was impaired as evidenced by an increase in the area under the glucose curve by 30% (Fig. 9C).

Expression of AKT2 mutants in skeletal muscle did not alter liver weights (Fig. 10A). Histological analysis of H&E-stained liver sections indicated increased lipid accumulation in *Pten*$^{+/+}$/AKT2^{K180A}.2A.GFP mice (Fig. 10B). Notably, staining of liver sections with Oil Red O and BODIPY493/503 revealed less hepatic lipids in *Pten*$^{+/+}$/myr-AKT2.2A.GFP mice and an increase in *Pten*$^{+/+}$/AKT2^{K180A}.2A.GFP mice relative to *Pten*$^{+/+}$/2A.GFP controls (Fig. 10C, D). Moreover, *Pten*$^{+/-}$/AKT2^{K180A}.2A.GFP mice had higher lipid content than *Pten*$^{+/-}$/2A.GFP mice (Fig. 10C, D). Importantly, these observations were confirmed by the quantification of BODIPY493/593-positive stained areas and triglyceride assays (Fig. 10E, F).

Taken together, the present data indicate that enhanced activity of AKT2 in skeletal muscle causes the reduction in hepatic lipid content in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice. The significance of AKT2 activity in skeletal muscle on accumulation of hepatic lipids is further underlined by the effects observed in *Pten*$^{+/+}$/*Akt2*$^{+/+}$ mice expressing AKT2 mutants in the skeletal muscle.

Discussion

With the present study, we have characterized the contribution of skeletal muscle to lipid accumulation in liver upon loss of PTEN. Previous reports proposed that lipid accumulation in PTEN-deficient liver is driven by enhanced *de novo* lipogenesis because of hyperactivated

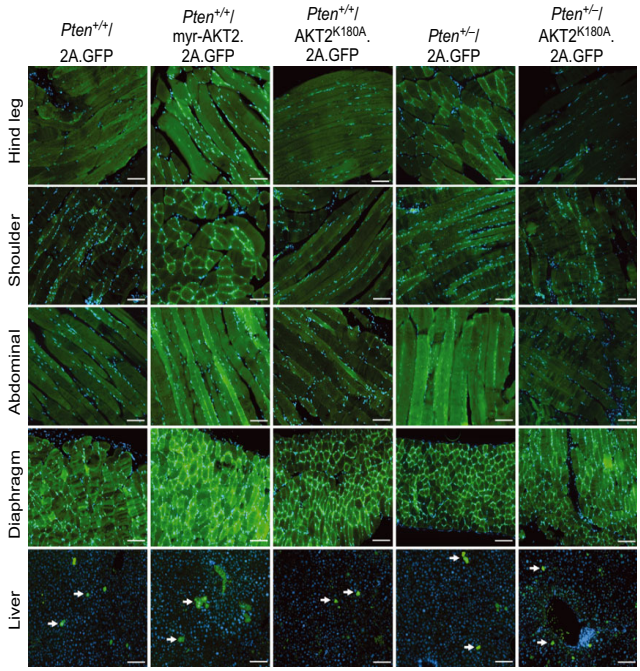


Fig. 8. Skeletal muscle-specific expression of 2A.GFP, myr-AKT2.2A.GFP and AKT2^{K180A}.2A.GFP in Pten$^{+/+}$/Akt2$^{+/+}$ and *Pten*$^{+/-}$*/Akt2* $^{+/+}$ mice Representative images of sections from indicated regions of skeletal muscle and liver stained for GFP (green) and DNA (blue). Arrows indicate GFP-positive hepatocytes. Scale bar = 100 μm.

AKT2 (19–21). Indeed, deletion of AKT2 was found to inhibit the development of hepatic steatosis in mouse models with hepatocyte-specific *Pten*-deficiency, in *leptin*-deficient mice and in mice fed a high-fat diet (21, 22). However, the accumulation of lipids in the liver might not only depend on hepatic AKT2 activity but also on AKT2 activity in peripheral insulin-sensitive tissues via systemic interactions. Indeed, in this study we found a reduced hepatic lipid content in mice with whole-body *Pten*-haplodeficiency despite hyperactivation of AKT in the liver. This observation is in contrast to hepatic steatosis reported in mice with hepatocyte-specific *Pten*-deficiency (19, 21, 34). The purpose of our subsequent analysis was to indentify a mechanism that reduces hepatic lipids in *Pten*$^{+/-}$*/Akt*$^{+/+}$ mice. *Pten*$^{+/-}$*/Akt2*$^{-/-}$ mice were used to determine if changes observed in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice depend on AKT2.

Pten$^{+/-}$*/Akt2*$^{+/+}$ mice showed significantly increased activation of AKT and upregulation of lipogenic genes in the liver, similar to mice with hepatocyte-specific *Pten* deletion (19, 21). This indicates that extrahepatic factors can prevent accumulation of lipids in the livers of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice in the presence of hyperactivated AKT2.

Physical exercise improves insulin sensitivity in skeletal muscle of human subjects and concomitantly leads to a reduction in hepatic lipid content (25). Because skeletal muscle of *Pten*$^{+/-}$ mice show increased insulin-stimulated glucose uptake we decided to test if enhanced insulin response of skeletal muscle could explain the reduced hepatic lipid content in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice (15). Indeed, *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice displayed an enhanced insulin response of skeletal muscle as evidenced by increased content of triglyceride and glycogen in fasted and insulin-stimulated mice respectively. Regulation of glycogen content is known to depend on AKT2 and AKT2 signalling was enhanced upon insulin stimulation. These results indicate that AKT2 in the skeletal muscle of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice reduces hepatic lipid content. Importantly, skeletal muscle-specific expression of dominant negative AKT2^{K180A} increased hepatic lipid content in *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice. Moreover, we found that skeletal muscle-specific expression of constitutively active myr-AKT2 and dominant negative AKT2^{K180A} in *Pten*$^{+/+}$*/Akt2*$^{+/+}$ mice reduced and increased hepatic lipid content, respectively. This observation shows that AKT2 activity in skeletal muscle plays a crucial role in regulating hepatic lipid content. In conclusion, these data support the view that an enhanced skeletal muscle insulin response mediated by AKT2 prevents accumulation of lipids in the liver of *Pten*$^{+/-}$*/Akt2*$^{+/+}$ mice.

Mice with whole-body *Pten*-haplodeficiency revealed that extrahepatic factors can override the lipogenic effects of hyperactivated AKT2 in the liver and that

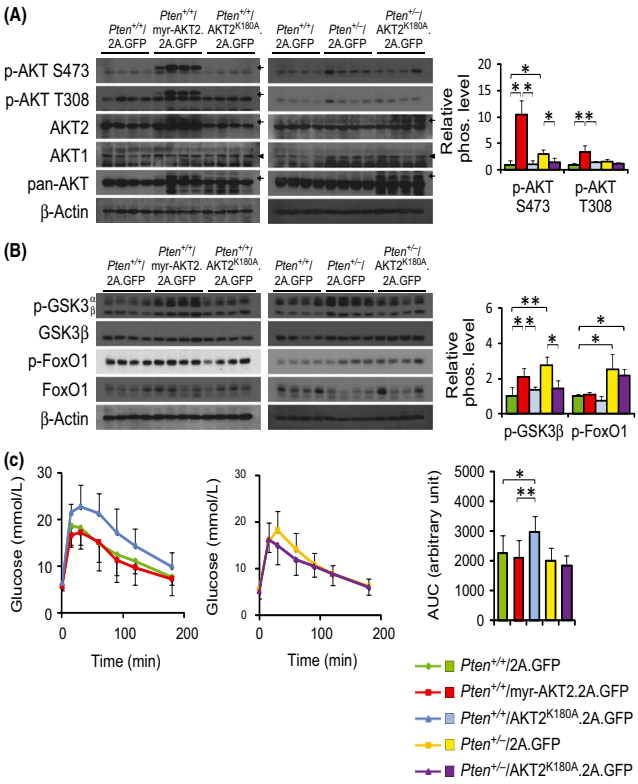


Fig. 9. Effects of skeletal muscle-specific expression of myr-AKT2 and AKT2^{K180A} on AKT signalling and glucose tolerance. (A) Western blot showing phosphorylation of AKT at S473 and T308 and AKT1, AKT2 and pan-AKT protein levels in skeletal muscle of fasted mice. Densitometric quantification is shown. Arrows indicate myr-AKT2 and AKT2^{K180A} transgenes, arrowheads indicate endogenous AKT1. n = 4/group. (B) Phosphorylation and protein levels of GSK3β and FoxO1 in skeletal muscle of fasted and insulin-stimulated mice. Densitometric quantification is shown. n = 3–4/group. (C) Blood glucose concentrations in fasted mice after intraperitoneal administration of glucose were measured at the indicated time points. Time course of changes in blood glucose concentration are separated according to genotype for a better overview. Respective AUCs are shown. n = 7–12/group; AUC, area under the curve; data are expressed as means ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$.

AKT2 activity in skeletal muscle plays a key role in the observed phenotype. The mechanistic link between AKT2 activity in skeletal muscle and hepatic metabolism remains to be elucidated and could be addressed by e.g. analysis of hepatic lipid synthesis and uptake, determining glucose disposal during hyperinsulinemic-euglycemic clamp as well as energy intake and expenditure in metabolic cages. Future studies with mice for tissue-specific deletion of PTEN and AKT2 would help to elucidate on how AKT2 in skeletal muscle affects hepatic lipid content.

PTEN and AKT2 activity in skeletal muscle are most likely not the only extrahepatic factor affecting lipid accumulation in the liver of *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice. This is supported by the observation that *Pten*$^{+/+}$/AKT2-^{K180A}.2A.GFP mice display higher hepatic lipid content compared to *Pten*$^{+/-}$/AKT2^{K180A}.2A.GFP mice which could be because of additional mechanisms influencing hepatic lipid content. Insulin-, PTEN- and/or AKT2-dependent processes such as adipose tissue lipolysis, energy expenditure in brown adipose tissue, exo- and endocrine function of the pancreas and food intake are likely to also affect hepatic metabolism in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice. For instance, adipose tissue lipolysis contributes significantly to hepatic lipid content (23). Disruption of mTORC1 complex, a component of insulin signalling pathway, in adipocytes leads to improved glycaemic control, enhanced energy expenditure in adipose tissue and reduced hepatic lipid content in mice (39). Adipocyte-specific deletion of *Pten* was shown to result in hyperactivated AKT and improved glycaemic control (40). Pancreas-specific deletion of *Pten* improves metabolic control and elevates hepatic AKT signalling (41). The improved metabolic control of mice with adipocyte- or pancreas-specific deletion of *Pten* suggest that hepatic lipid content is also affected in these mice, but the hepatic lipid content has not been addressed in these studies (40, 41). The

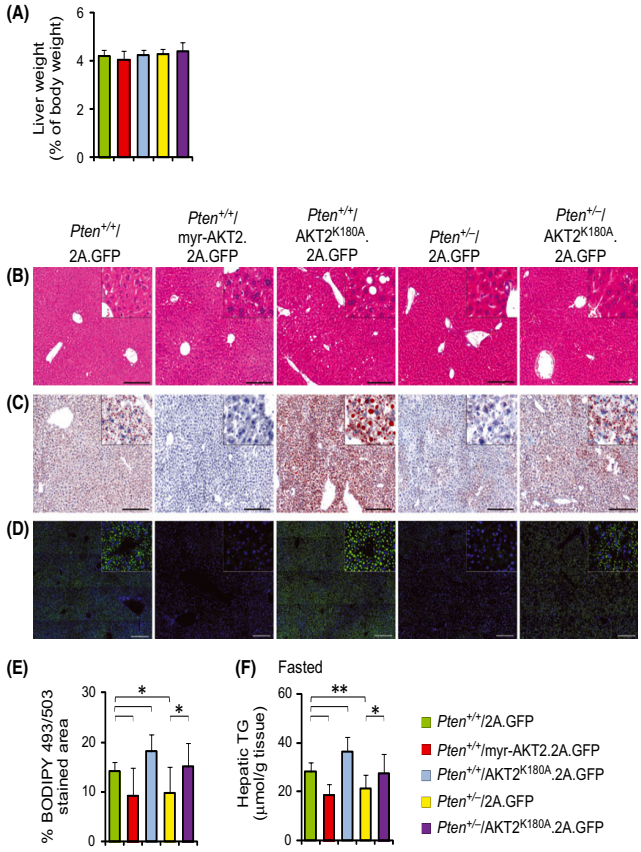


Fig. 10. Skeletal muscle-specific expression of myr-AKT2 and AKT2^{K180A} affects hepatic lipid content. (A) Liver to body weight ratios. (B, C, D) Representative images of liver sections stained with H&E (B), for lipids with Oil Red O (C) and for lipids and DNA with BODPIY493/503 (green) and DAPI (blue), respectively (D). Image inlays show part of the depicted area at higher magnification. (E) Relative BODIPY493/503-stained areas. (F) Hepatic triglyceride content in fasted mice. TG, triglycerides; scale bar = 200 µm; n = 7–12/group; data are expressed as means ± SD; *$P < 0.05$, **$P < 0.01$.

individual role of PTEN and AKT2 in additional tissues such as pancreas and adipose tissue on hepatic metabolism could be masked and difficult to characterize in mice with whole-body deletion of *Pten* and *Akt2*. Hence, mice with specific deletion of *Pten* and *Akt2* in respective tissues are more suitable to study the effects of additional PTEN- and AKT2-dependent processes on hepatic metabolism in the future.

While *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice showed normal blood glucose at 2 h after a single intraperitoneal injection of glucose during the glucose tolerance test, the blood glucose of refed *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice was significantly increased after 2 h. Mice were given food *ad libitum* during fasting-refeeding experiment. This extended challenge could be the reason for elevated blood glucose concentration of fasted-refed *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice.

Pten$^{+/-}$/*Akt2*$^{-/-}$ mice showed increased random fed and fasted-refed blood glucose. But fasted blood glucose, glucose tolerance and skeletal muscle glycogen content of *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice were similar to wild-type mice, despite abrogated basal and insulin-stimulated AKT phosphorylation in the liver and skeletal muscle. A weak correlation between AKT activation and insulin action has been observed previously (42, 43). The weak correlation of AKT phosphorylation and physiological effects in *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice might be in part because of redundancy of AKT isoforms (10). The high level of p-AKT T308 in the liver of fasted *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice points to a compensation by AKT1. Similar effects were shown previously in mice with liver-specific deletion of *Akt2* that are deficient for leptin or fed with a high-fat diet (22). A potential compensation by AKT1 and/or AKT3 upon loss of AKT2 might be more pronounced on the background of *Pten*-haplodeficiency in our mouse model. For instance, the level of pGSK3β in the liver of fasted *Pten*$^{+/-}$/*Akt2*$^{+/+}$

and *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice is similar. We speculate that this could be a result of increased AKT1 activation.

Loss of AKT2 in a *Pten*-haplodeficient background mainly reduces p-AKT S473 and to a lesser extent p-AKT T308. This result indicates that regulation of p-AKT S473 and T308 might be AKT isoform-specific and differentially dependent on PTEN. This is an interesting observation but its significance remains unclear as the tissue-specific function(s) of individual AKT isoforms are not fully understood. Loss of AKT2, however, results in insulin resistance and glucose intolerance. Together with the observed reduction in mainly p-AKT S473 our finding indicates that insulin sensitivity and systemic regulation of glucose homoeostasis may be more dependent on p-AKT S473 than on p-AKT T308.

The expression of lipogenic genes in the liver of *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice was at similarly high levels as observed in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice. This is in line with previous findings showing that the regulation of lipogenic genes by AKT2 is context-dependent (19, 21, 22). While the expression of lipogenic genes was found to be dependent on AKT2 in mice with hepatocyte-specific *Pten*-deficiency and *leptin*-deficiency, their expression was not altered in *Akt2*-deficient mice fed a normal chow or a high-fat diet enriched in simple carbohydrates (Surwit diet) (19, 21, 22). It is possible that AKT1 or other PI3K-dependent kinases such as PKCλ/ζ could compensate the loss of AKT2 and induce expression of lipogenic genes in fasted *Pten*$^{+/-}$/*Akt2*$^{-/-}$ mice (44).

Pten$^{+/-}$/*Akt2*$^{-/-}$ mice show increased random fed and fasted-refed blood glucose level and abrogated activation of AKT in skeletal muscle. Similarly, *Akt2*$^{-/-}$ mice have increased blood glucose level and reduced AKT activation in skeletal muscle. However, hepatic lipid content of both *Pten*$^{+/-}$/*Akt2*$^{-/-}$ and *Akt2*$^{-/-}$ mice is reduced as in *Pten*$^{+/-}$/*Akt2*$^{+/+}$ mice. This indicates that AKT2 in hepatocytes is required but not sufficient for accumulation of lipids in the liver of *Pten*-haplodeficient mice. This observation also supports previous reports showing that AKT2 has a hepatocyte-autonomous role in accumulation of hepatic lipids (21, 22).

Recent studies have suggested that insulin resistance of skeletal muscle is a central factor in the development of NAFLD (7, 24, 25). Lifestyle changes were shown to be effective in NAFLD treatment (45). For instance, 2 weeks of diet and exercise therapy in diabetic patients reduced hepatic lipid content by 27% (45, 46). However, lifestyle changes are often difficult to achieve (47). Our data show that enhanced insulin response in skeletal muscle mediated by AKT2 reduces hepatic lipid content. Thus, pharmacological activation of AKT2 specifically in skeletal muscle might be an effective option for treatment of NAFLD.

Acknowledgements

We are grateful to Pier P. Pandolfi (Beth Israel Deaconess Medical Center, Harvard Medical School, USA) for providing *Pten*$^{+/-}$ mice and Nicholas K. Tonks (Cold Spring Harbor Laboratory, USA) for providing antibodies against PTEN. We thank Heidi Seiler, Josephine Juettner, Sandrine Bichet, Peter Cron and Arno Doelemeyer for excellent technical support and Patrick King for editing the manuscript.

S.M.S. and O.T. were supported by the Swiss SystemsX.ch initiative LiverX of the Competence Center for Systems Physiology and Metabolic Diseases. S.M.S. was supported by the Olga Mayenfisch Foundation and the Forschungskredit of the University of Zurich. O.T. was supported by the Amélie Waring Foundation.

Conflict of interest: The authors do not have any disclosures to report.

References

1. Browning JD, Szczepaniak LS, Dobbins R, *et al.* Prevalence of hepatic steatosis in an urban population in the United States: Impact of ethnicity. *Hepatology* 2004; **40**: 1387–95.
2. Schwimmer JB, Deutsch R, Kahen T, *et al.* Prevalence of fatty liver in children and adolescents. *Pediatrics* 2006; **118**: 1388–93.
3. Tuyama AC, Chang CY. Non alcoholic fatty liver disease. *J Diabetes* 2012; **4**: 266–80.
4. Angulo P, Lindor KD. Non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 2002; **17**: S186–90.
5. Cohen JC, Horton JD, Hobbs HH. Human fatty liver disease: Old questions and new insights. *Science* 2011; **332**: 1519–23.
6. Brown MS, Goldstein JL. Selective vs. total insulin resistance: a pathogenic paradox. *Cell Metab* 2008; **7**: 95–6.
7. Petersen KF, Dufour S, Savage DB, *et al.* The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. *Proc Natl Acad Sci U S A* 2007; **104**: 12587–94.
8. Semple RK, Sleigh A, Murgatroyd PR, *et al.* Postreceptor insulin resistance contributes to human dyslipidemia and hepatic steatosis. *J Clin Invest* 2009; **119**: 315–22.
9. Schultze SM, Hemmings BA, Niessen M, Tschopp O. PI3K/AKT, MAPK and AMPK signalling: protein kinases in glucose homeostasis. *Expert Rev Mol Med* 2012; **14**: e1.
10. Schultze SM, Jensen JR, Hemmings BA, Tschopp O, Niessen M. Promiscuous affairs of PKB/AKT isoforms in metabolism. *Arch Physiol Biochem* 2011; **117**: 70–7.
11. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007; **129**: 1261–74.
12. Cho H, Mu J, Kim JK, *et al.* Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* 2001; **292**: 1728–31.
13. Dummler B, Tschopp O, Hynx D, *et al.* Life with a single isoform of Akt: mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. *Mol Cell Biol* 2006; **26**: 8042–51.
14. Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP. Pten is essential for embryonic development and tumour suppression. *Nat Genet* 1998; **19**: 348–55.
15. Wong J, Kim P, Peacock J, *et al.* Pten (phosphatase and tensin homologue gene) haploinsufficiency promotes insulin hypersensitivity. *Diabetologia* 2007; **50**: 395–403.

APPENDIX

16. Blumenthal GM, Dennis PA. PTEN hamartoma tumor syndromes. *Eur J Hum Genet* 2008; **16**: 1289–300.
17. Pal A, Barber TM, van de Bunt M, *et al.* PTEN mutations as a cause of constitutive insulin sensitivity and obesity. *N Engl J Med* 2012; **367**: 1002–11.
18. Vinciguerra M, Veyrat-Durebex C, Moukil MA, *et al.* PTEN down-regulation by unsaturated fatty acids triggers hepatic steatosis via an NF-kBp65/mTOR-dependent mechanism. *Gastroenterology* 2008; **134**: 268–80.
19. Stiles B, Wang Y, Stahl A, *et al.* Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity. *Proc Natl Acad Sci U S A* 2004; **101**: 2082–7.
20. Horie Y, Suzuki A, Kataoka E, *et al.* Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas. *J Clin Invest* 2004; **113**: 1774–83.
21. He L, Hou X, Kanel G, *et al.* The critical role of AKT2 in hepatic steatosis induced by pten loss. *Am J Pathol* 2010; **176**: 2302–8.
22. Leavens KF, Easton RM, Shulman GI, Previs SF, Birnbaum MJ. Akt2 is required for hepatic lipid accumulation in models of insulin resistance. *Cell Metab* 2009; **10**: 405–18.
23. Donnelly KL, Smith CI, Schwarzenberg SJ, *et al.* Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* 2005; **115**: 1343–51.
24. Flannery C, Dufour S, Rabol R, Shulman GI, Petersen KF. Skeletal muscle insulin resistance promotes increased hepatic de novo lipogenesis, hyperlipidemia, and hepatic steatosis in the elderly. *Diabetes* 2012; **61**: 2711–7.
25. Rabol R, Petersen KF, Dufour S, Flannery C, Shulman GI. Reversal of muscle insulin resistance with exercise reduces postprandial hepatic *de novo* lipogenesis in insulin resistant individuals. *Proc Natl Acad Sci U S A* 2011; **108**: 13705–9.
26. Andjelkovic M, Jakubowicz T, Cron P, *et al.* Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors. *Proc Natl Acad Sci U S A* 1996; **93**: 5699–704.
27. Andjelkovic M, Alessi DR, Meier R, *et al.* Role of translocation in the activation and function of protein kinase B. *J Biol Chem* 1997; **272**: 31515–24.
28. Grieger JC, Choi VW, Samulski RJ. Production and characterization of adeno-associated viral vectors. *Nat Protocols* 2006; **1**: 1412–28.
29. Wang Z, Zhu T, Qiao C, *et al.* Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nat Biotech* 2005; **23**: 321–8.
30. Ehses J, Meier D, Wueest S, *et al.* Toll-like receptor 2-deficient mice are protected from insulin resistance and beta cell dysfunction induced by a high-fat diet. *Diabetologia* 2010; **53**: 1795–806.
31. Spandidos A, Wang X, Wang H, Seed B. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucleic Acids Res* 2010; **38**(Suppl. 1): D792–9.
32. Tschopp O, Yang ZZ, Brodbeck D, *et al.* Essential role of protein kinase B gamma (PKB gamma/Akt3) in postnatal brain development but not in glucose homeostasis. *Development* 2005; **132**: 2943–54.
33. Buzzi F, Xu L, Zuellig RA, *et al.* Differential effects of protein kinase B/Akt isoforms on glucose homeostasis and islet mass. *Mol Cell Biol* 2010; **30**: 601–12.
34. Piguet A-C, Stroka D, Zimmermann A, Dufour J-F. Hypoxia aggravates non-alcoholic steatohepatitis in mice lacking hepatocellular PTEN. *Clin Sci* 2009; **118**: 401–10.
35. Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J Clin Invest* 2006; **116**: 615–22.
36. Delibegovic M, Bence KK, Mody N, *et al.* Improved glucose homeostasis in mice with muscle-specific deletion of protein-tyrosine phosphatase 1B. *Mol Cell Biol* 2007; **27**: 7727–34.
37. Garofalo RS, Orena SJ, Rafidi K, *et al.* Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKBbeta. *J Clin Invest* 2003; **112**: 197–208.
38. Brozinick JT, Roberts BR, Dohm GL. Defective signaling through Akt-2 and -3 But Not Akt-1 in insulin-resistant human skeletal muscle: potential role in insulin resistance. *Diabetes* 2003; **52**: 935–41.
39. Polak P, Cybulski N, Feige JN, *et al.* Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration. *Cell Metab* 2008; **8**: 399–410.
40. Kurlawalla-Martinez C, Stiles B, Wang Y, *et al.* Insulin hypersensitivity and resistance to streptozotocin-induced diabetes in mice lacking PTEN in adipose tissue. *Mol Cell Biol* 2005; **25**: 2498–510.
41. Tong Z, Fan Y, Zhang W, *et al.* Pancreas-specific Pten deficiency causes partial resistance to diabetes and elevated hepatic AKT signaling. *Cell Res* 2009; **19**: 710–9.
42. Hoehn KL, Hohnen-Behrens C, Cederberg A, *et al.* IRS1-independent defects define major nodes of insulin resistance. *Cell Metab* 2008; **7**: 421–33.
43. Xu L, Spinas GA, Niessen M. ER stress in adipocytes inhibits insulin signaling, represses lipolysis, and alters the secretion of adipokines without inhibiting glucose transport. *Horm Metab Res* 2010; **42**: 643–51.
44. Taniguchi CM, Kondo T, Sajan M, *et al.* Divergent regulation of hepatic glucose and lipid metabolism by phosphoinositide 3-kinase via Akt and PKC. *Cell Metab* 2006; **3**: 343–53.
45. Harrison SA, Day CP. Benefits of lifestyle modification in NAFLD. *Gut* 2007; **56**: 1760–9.
46. Tamura Y, Tanaka Y, Sato F, *et al.* Effects of diet and exercise on muscle and liver intracellular lipid contents and insulin sensitivity in type 2 diabetic patients. *J Clin Endocrinol Metab* 2005; **90**: 3191–6.
47. Hill JO. Can a small-changes approach help address the obesity epidemic? A report of the Joint Task Force of the American Society for Nutrition, Institute of Food Technologists, and International Food Information Council. *Am J Clin Nutr* 2009; **89**: 477–84.