Insulin and cellular stress induced glucose uptake in 3T3-L1 adipocytes

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Chapter I

Insulin induced signal-transduction pathways in 3T3-L1 adipocytes.

One of the main functions of the hormone insulin is in maintaining whole-body glucose homeostasis, keeping the plasma glucose levels in a narrow range around 5 mM in normal individuals despite periods of feeding and fasting [1-3]. Loss of this ability can lead to a wide range of disease states such as arteriosclerosis, cardio-vascular diseases, diabetic foot, retinopathy, nephropathy and diabetes. Two separate mechanisms can lead to loss of metabolic control. First there is loss of the hormone insulin due to the destruction or incapacitation of the β–cell population in the Islets of Langerhans. This type of affliction is seen in Type I diabetes mellitus and other states of insulinopenia [4-6]. Another mechanism leading to poor metabolic control is loss of sensitivity of the main insulin-responsive target organs such as liver, muscle and adipose tissue to the actions of the hormone insulin. This situation is characteristic for the metabolic syndrome [7;8]. Initially this loss of insulin sensitivity is met by an increased production of insulin by the β-cell population until the system can no longer provide adequate amounts of insulin, blood glucose homeostasis is lost and full-blown type II diabetes mellitus has been established [9;10]. In the western world, type II diabetes is rapidly reaching epidemic proportions due to excessive caloric intake combined with a profound lack of exercise [11-14]. Loss of insulin-sensitivity can be caused by a combination of defects occurring in the insulin-induced signal-transduction pathway. This is underlined by the complex interplay between genetic and environmental factors impinging on, and ultimately leading to the onset of type II diabetes [15-18]. Due to this interplay, and the fact that the β-cell population can go a long way to meet the increased demand of insulin in the body, the onset of type II diabetes was traditionally at a later age, giving rise to its popular name “sugar of the elderly”. It is worthwhile to keep in mind however, that with the present day life style the age of onset has decreased sharply making this popular name misleading in grasping the severity of the epidemic [19-21].

The Insulin Receptor

Insulin mediates its cellular functions through binding to its cognate receptor, which has been identified over thirty years ago [22]. The central role of insulin-signalling and the importance of the Insulin Receptor in mediating both mitogenic and metabolic actions of the hormone insulin is illustrated by the phenotype of the Insulin Receptor knock-out mice. These mice are born at term with only a slight growth retardation (~10%), but rapidly develop lethal postnatal diabetes [23-25]. There are several

inherited afflictions in humans associated with defects in the Insulin Receptor, such as leprechaunism, Rabson-Mendenhall syndrome and type A syndrome of insulin resistance [26-29].
The Insulin Receptor (IR) and its closest homologues : the IGF1-receptor (IGF-IR) and the Insulin-Related Receptor (IRR) belong to a super family of tyrosine kinase receptors involved in mammalian growth, metabolism and reproduction [30-32]. Aside from its expression in well-known insulin responsive target tissues such as muscle, adipose tissue and liver, functional Insulin Receptor signalling has also been found in the β-cell and the brain [23]. Tissue-specific ablation of the Insulin Receptor in these tissues illustrates both canonical and non-canonical insulin-target tissues can contribute to insulin-resistance [33].
In its native state the receptor exists as a tetramer with two membrane spanning β–chains which harbour the intracellular tyrosine kinase domain and two extracellular α-chains which form the main part of the insulin-binding domain. The two β-chains and the α-β chains are cross linked to one-another by several disulfide bonds [32].
When insulin binds into the tunnel formed by the two α-chains, the relative juxtaposition of the two intracellular β-chains alters [34;35]. This induces ATP-binding and activation of the intracellular tyrosine kinase domain [36-38]. Subsequently the tyrosine kinase domain phosphorylates the intracellular β–chain on several tyrosine-residue clusters. Phosphorylation of the kinase regulatory domain (Y^{1146}, Y^{1150} and Y^{1151}) further enhances insulin receptor tyrosine kinase activity, whereas phosphorylation of the juxtamembrane tyrosine residues (Y^{953}, Y^{960} and Y^{972}) functions as docking sites for a wide range of proteins [39]. The C-terminal tyrosine-cluster, Y^{1316} and Y^{1322} serve to restrain mitogenic signalling of the insulin receptor [40-44]. Indeed, Y^{1316} is not conserved between the IR and the more mitogenic IGF-IR. Furthermore, different phenotypes of the IR and IGF-IR knock-out mice illustrate the predominant involvement of insulin-signalling in metabolic regulation and IGF-signalling in cellular growth [24;45-48]. Several intracellular signal-transduction pathways emanate from the activated Insulin Receptor, these signalling axes will be considered in detail with a focus on the insulin-responsive adipocyte.

The Insulin Receptor Substrate proteins
A range of adaptor proteins associate with the activated Insulin Receptor. Amongst these are Grb10, isoforms of Shc, $p60^{dok}$, pp120 Ceacam-1, Gab-1, APS and the IRS-protein family [49-53]. Whereas Grb10-binding inhibits insulin signalling in adipocytes altogether, pp120 Ceacam-1 binding to Y^{1316} only hampers mitogenic signalling [50;54-56].

Best described thus far are the IRS proteins, which form bona fide signalling platforms in the adipocyte. The different IRS homologues, named IRS-1,-2,-3 and –4 are not related by extensive amino-acid sequence identity but are similar with respect to their general architecture [57-63]. They are composed of an N-terminal PH-domain which binds membrane phospholipids and/or mediates protein-protein interactions [64-66]. The PH-domain is followed by a PTB domain which interacts with the phosphorylated $NPEY^{960}$-motif located in the juxtamembrane region of the insulin-receptor β-chain [59;67;68]. The C-terminal tail is less conserved and contains multiple potential tyrosine phosphorylation motifs that can bind to specific SH2-domain containing proteins such as the p85 regulatory subunit of PI-3'kinase, Grb-2, SHP-2 , Fyn, Crk, Csk and phospholipase Cγ as well as proline-rich regions capable of interacting with SH3- or WW-domain containing proteins such as Nck [49;69-72]. The middle of IRS-2 comprises a unique region comprising amino-acids 591-786 that interacts specifically with the regulatory loop of the insulin receptor tyrosine-kinase [73;74].
Studies in knock-out mice and cell-lines suggest that the IRS proteins serve complementary, rather than redundant roles. Several factors contribute to this differential signalling, such as differences in tissue and developmental expression, associating proteins, and subcellular localisation [70;75-78].
Thus, IRS-1 knock-out mice show growth retardation and insulin-resistance in peripheral tissues, but do not develop overt diabetes [79;80]. Conversely IRS-2 knockout mice do develop type II diabetes, primarily caused by a failure in compensatory β-cell hyperplasia, aside from peripheral insulin-resistance [81]. IRS-3 and –4 knockout mice have near normal growth and metabolism [82;83].
In rat or mouse adipocytes, only IRS-1, -2 and –3 are expressed [76]. In these cells, IRS-1 is the predominant target of insulin-signalling : IRS-1 levels are roughly tenfold upregulated during adipogenesis, whereas IRS-2 is increased only twofold [84]. Second, expression of a ribozyme directed against IRS-1 profoundly decreases insulin-stimulated GLUT4 translocation [85]. And third, this phenotype is reiterated in adipocytes obtained from IRS-1 knock-out mice [86].
As far as IRS-3 is concerned, there is no human orthologue of this protein [87]. However, in adipocytes derived from IRS-1 knockout mice, IRS-3 and not IRS-2, associated with PI-3'kinase after insulin-stimulation [88;89]. Thus, in mouse and rat adipocytes there is a redundancy between IRS-1 and –3.
In addition to tyrosine phosphorylation, IRS proteins also undergo serine/threonine phosphorylation which provides a negative feedback to insulin signalling and serves as an integration point for cross-talk from

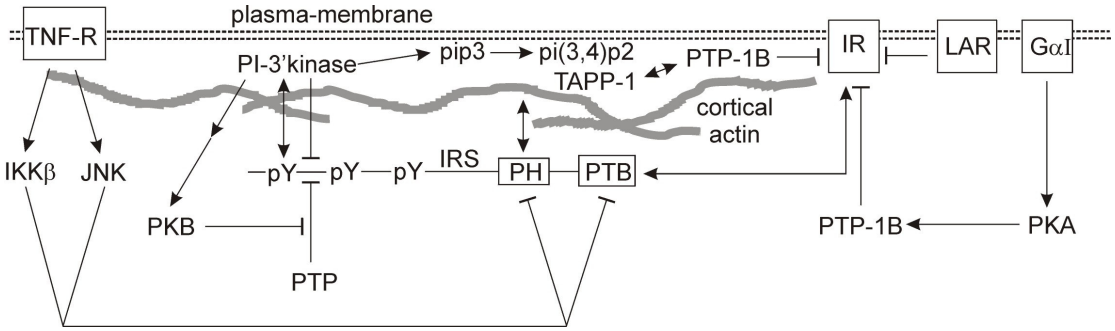

Fig. 1 Regulation routes of IR signalling
Signalling routes in 3T3-L1 adipocytes involved in regulation of IR-signalling. Activation steps are indicated by arrows, inhibitory steps are indicated by bars, and associations are depicted by double arrows. TAPP-1 associates directly with the pip_3 breakdown-product $pi(3,4)p_2$ acting as a PTP-1B scaffold. Grey lines represent the cortical actin structure (see the next chapter for more information on this adipocyte cell-morphological structure). The IRS proteins are shown in more detail, represented by a line with the C-terminal tyrosine-residues and the N-terminal PTB- and PH-domain indicated. The full name of all protein components can be found in the list of abbreviations at the end of this thesis.

other pathways [1]. The link between FFAs, prolonged insulin-treatment or TNF-α and increased IRS serine phosphorylation has been thoroughly described [90-93]. For example : disruption of the TNF receptor reduces IRS Ser/Thr-phosphorylation and improves insulin sensitivity [94;95]. Two main signalling branches emanating from the TNF-receptor are involved in mediating this effect (Fig. 1). The first is the activation of IκB kinase-β, which is also involved in FFA-induced insulin-resistance [90]. Consequently, treatment with salicylates or heterozygous disruption of IKK-β confers protection against obesity-induced diabetes [96-99]. The other branch involves c-Jun N-terminal kinase (JNK)[100;101]. This archetypal stress-activated kinase phosphorylates IRS-1 on S^{307} in the PTB-domain thereby disrupting IR-IRS-1 association [102;103].
With respect to negative feedback-loops, several insulin-induced kinases have been implicated, such as ERK-1/-2, PI-3'kinase, PKB, PKC-λ/ζ , GSK-3β and mTOR (Fig. 2 and 3)[104-114].
For most the outcome is deleterious for insulin signalling, as they impede binding of downstream effectors such as PI-3'kinase or hamper IR-IRS association. However, PKB- or mTOR- mediated phosphorylation of S^{265}, S^{302}, S^{325} and S^{358} protects mouse IRS-1 from the activity of tyrosine-phosphatases and thus potentates IRS-function (Fig. 1)[115-117].
In order to ensure a rapid metabolic response, the IRS proteins have to be engaged rapidly and specifically with the activated insulin-receptor. In order to achieve this, these soluble proteins are associated with filamentous cortical-actin structures running parallel to the plasma

membrane. By sliding along these structures the movement of the IRS-proteins is limited in a two-dimensional space (Fig. 1). As a consequence the efficiency of coupling to the activated insulin-receptor is increased and concomitantly PI-3' kinase (a downstream target) is localised to the plasma-membrane [118-123]. Indeed, disassembly of the actin network using cytochalasin D prevents insulin-induced glucose transport and PI-3'kinase signalling [124;125]. Furthermore, a GFP-tagged PI(3,4,5)P_3-binding protein predominantly localises at the plasma-membrane in adipocytes stimulated with insulin [126;127]. Aside from the above-mentioned interference with IR-IRS interactions, Ser/Thr phosphorylation of the IRS-proteins can also disrupt the cytoskeletal localisation, thereby inducing insulin-resistance [128;129].

Phosphatidyl-Inositol 3' kinase

A crucial effector binding to tyrosine phosphorylated IRS is Phosphatidyl-Inositol 3' kinase (PI-3'kinase) (Fig. 2)[130]. This protein is a member of a super family of lipid-kinases, which also includes bona fide protein kinases such as ATM, ATR and mTOR [131;132]. Actually, protein kinase activity has also been reported for PI-3' kinase, and is involved both in autophosphorylation as well as negative feedback control of the IRS-proteins [105;106;108;133-135].

Three classes of PI-3' kinases are defined on basis of their primary structure and substrate specificity [136-138] : Class I PI-3'kinases generate all three types of phosphoinositides and are activated by receptor tyrosine kinases and G-protein-coupled receptors. These kinases consist of heterodimeric enzymes composed of regulatory and catalytic subunits and are further subdivided in two main classes. Subclass Ia includes the catalytic p110α, p110β and p110δ subunits and are regulated by binding to either phospho-tyrosine or to proline-rich domains [139-141]. These catalytic subunits consist of a C-terminal catalytic domain, a Phosphatidyl-Inositol Kinase (PIK) domain, a N-terminal Ras-binding domain and a regulatory-subunit binding domain. Whereas the α- and β-isoforms are ubiquitously expressed, expression of the δ-isoform is limited to haematopoietic cells [141]. Subclass Ib only contains the regulatory subunit p101 and the catalytic subunit p110γ. This subclass mediates signalling of GPCR through binding of Gβ/γ [142;143].

Class II PI-3'kinases generate PI(3)P and PI(3,4)P_2. This class consists of two subclasses (α and β) which are characterised by a C-terminal C2-domain [131]. These domains were originally observed in PKC isoforms where they mediate phospholipid binding in the presence of Ca^{2+} [144].

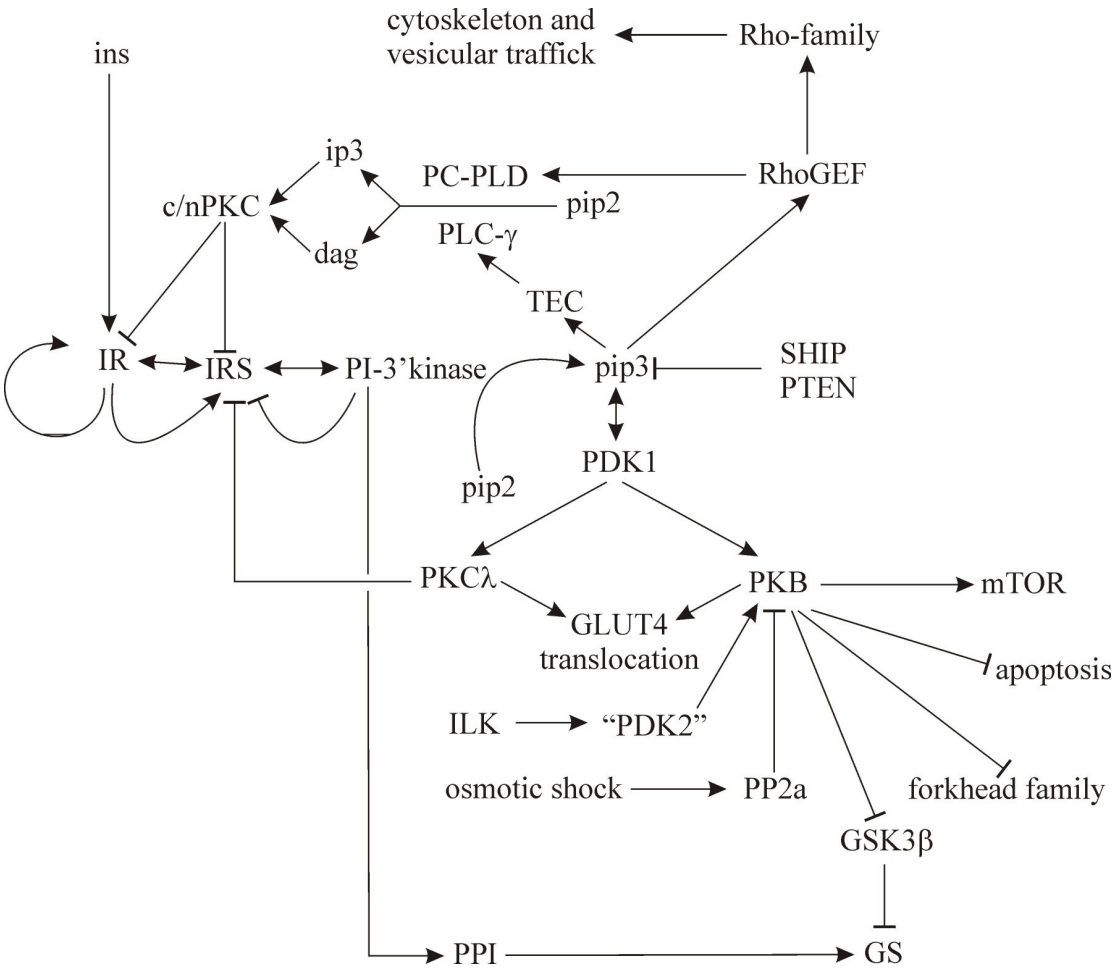

Fig. 2 PI-3'kinase signalling routes
Insulin-induced signalling routes in 3T3-L1 adipocytes. Activation steps are indicated by arrows, inhibitory steps are indicated by bars, and associations are depicted by double arrows. The full name of all protein components can be found in the list of abbreviations at the end of this thesis.

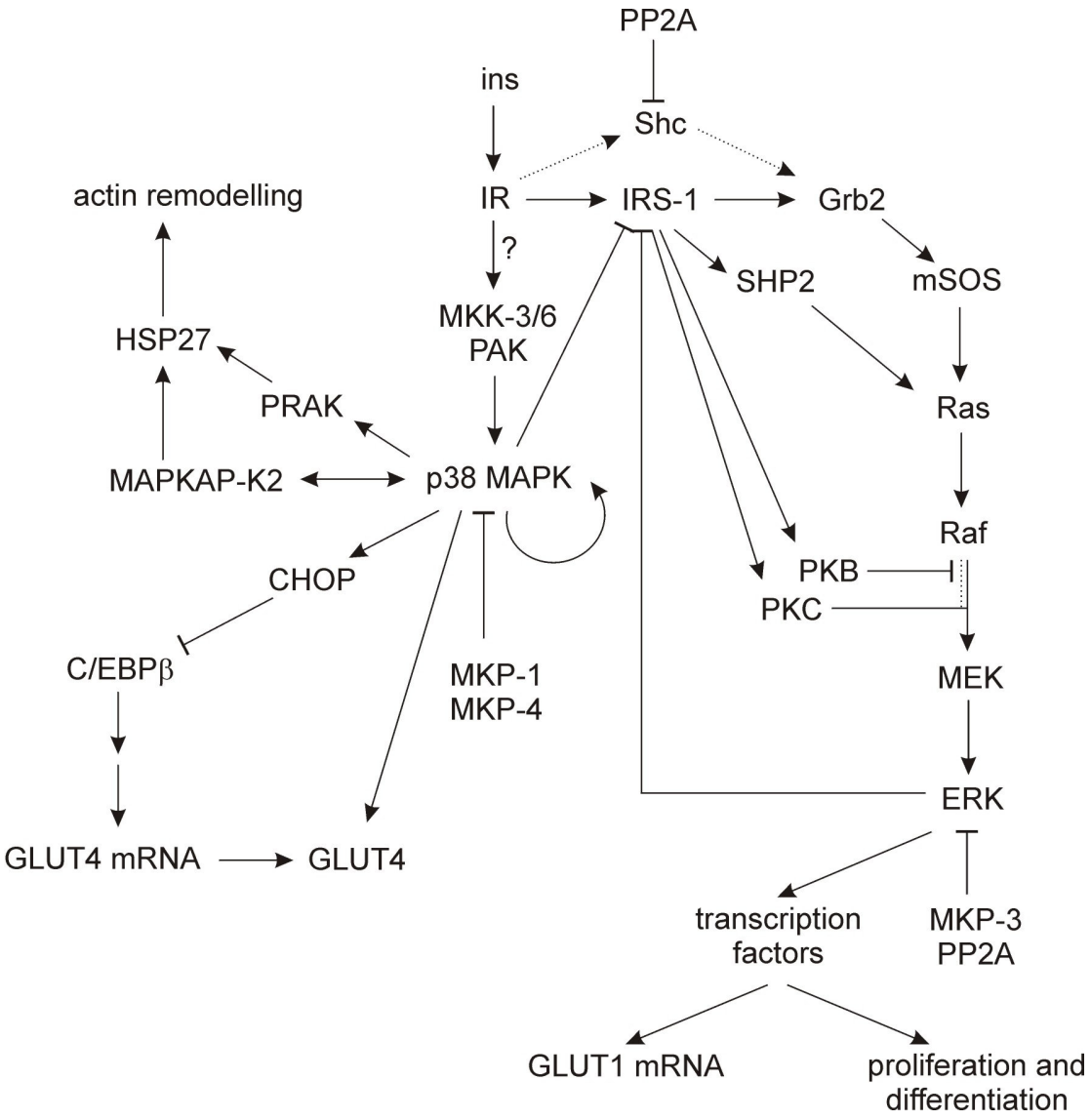

Fig. 3 MAPK signalling routes
Insulin-induced signalling routes in 3T3-L1 adipocytes. Activation steps are indicated by arrows, inhibitory steps are indicated by bars. Dashed lines illustrate MAPK signalling-routes that are disconnected during adipogenesis (see also next chapter). The full name of all protein components can be found in the list of abbreviations at the end of this thesis.

Class III PI-3'kinases only generate PI(3)P [145]. Because this is the only class present in yeast (Vps34p) it is thought to represent the primordial PI-3' kinase. These enzymes consist of C-terminal catalytic and PIK domains and are also sensitive to wortmannin [145;146]. Their regulatory subunit contains an N-terminal myristoylation signal, a Ser/Thr kinase domain, a series of leucine-rich repeats and a C-terminal WD motif [147]. This class of PI-3'kinases plays an important role in vesicular trafficking, endocytosis and osmoregulation [148]. Several recent manuscripts indicate a major role for PI(3)P in insulin-induced GLUT4 translocation [149;150].

The regulatory subunits of Class Ia PI-3'kinases form a complex protein family consisting of five regulatory subunits derived from three genes. Different, but highly related genes encode p85α and p85β [134;151]. Both contain two C-terminal SH2-domains followed by N-terminal SH3 and BCR-homology domains flanked by proline-rich regions. Alternative splicing or differential transcriptional initiation of p85α yields as53/p55α and p50α [152-154]. A third gene encodes $p55^{pik}$/p55γ [155]. Studies with knock-out mice show that p85α and its splice variants are responsible for 75% of the insulin induced PI-3'kinase activity [156;157]. In insulin-signalling, PI-3' kinase is activated by the association of the SH2-domains of p85-regulatory subunit with tyrosine-phosphorylated pYMXM and pYXXM motifs in the IRS-proteins [158;159]. The association between p85 and IRS and between p85 and p110 enhances the catalytic activity of p110 [134;158;160] Active PI-3' kinase subsequently phosphorylates inositol lipids at the D3 position of the inositol ring to generate the 3'-phosphoinositides PI(3)P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ [161;162]. Several observations illustrate the importance of PI-3' kinase in insulin-signalling. First, the fungal metabolite wortmannin irreversibly inhibits the catalytic subunit of Class I PI-3'kinases at low nanomolar concentrations by Schiff base formation with a lysine in the kinase domain [163-166]. The structurally unrelated LY294002, a pharmaceutical compound, is also inhibitory but reversibly and at micromolar concentrations [167;168]. Application of these compounds potently inhibits insulin-induced GLUT4 translocation and glucose uptake [169-172]. Second, microinjection or ectopic expression of a dominant-negative p85 incapable of associating with phosphotyrosine residues completely blocks insulin-induced GLUT4 translocation [173;174]. Intriguingly though, a mouse lacking p85β, the p85α isoform alone or heterozygous knock-out of all p85α-isoforms shows improved insulin-sensitivity [156;175;176]. This is due to the stoichometry of the regulatory p85-subunit versus the catalytic p110-subunit (Fig. 4).

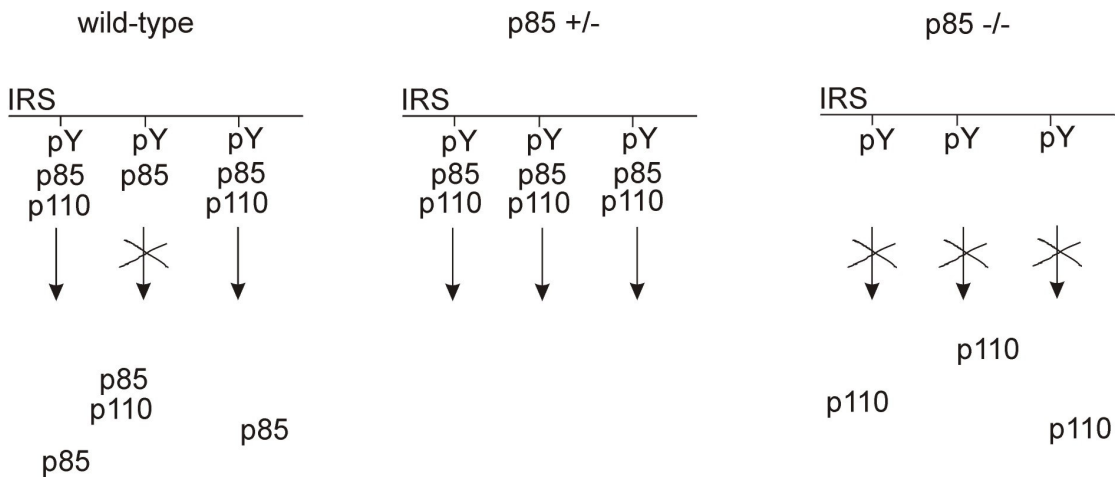

Fig. 4 Modulatory effects of p85 subunit stoichometry on PI-3'kinase activity. The C-terminal phosphorylated tyrosine-residues of the IRS-proteins are represented by the pY's on a line. The 85kDa regulatory(p85)- and 110kDa catalytic(p110) are depicted either in their monomeric form or as a complex. Functional PI-3'kinase signalling is depicted as an arrow. When the arrow is crossed out, no PI-3'kinase signalling occurs.

The former is expressed at higher levels than the latter. Consequentially, in a wild-type cell part of the available phosphotyrosine residues will be occupied by non-active p85-subunits, whereas in a heterozygous knock-out these non-functional p85-subunits will be replaced by functional PI-3' kinase instead [156;157].
Although PI-3' kinase activity is essential for insulin-induced GLUT4 translocation, it has long been appreciated that in itself this not enough. For example, the insulin, IGF-1 and IL-4 receptor and integrin all activate PI-3' kinase through the IRS-proteins. However, only insulin induces GLUT4 vesicle translocation in the adipocyte [70;177;178]. Furthermore, several stimuli, amongst which osmotic shock and guanosine 5'-O-3-thiophosphate (GTPγS) stimulate GLUT4 translocation and glucose uptake in adipocytes without concomitant PI-3'kinase activation [179-181]. A further illustration is provided by the application of cell-permeable $PI(3,4,5)P_3$-analogues. Thus, treatment of 3T3-L1 adipocytes with these analogues does not induce GLUT4 translocation. Yet, this compound restores GLUT4 translocation when applied to insulin-stimulated cells treated wortmannin to inhibit PI-3'kinase, demonstrating its functionality and illustrating the requirement for additional insulin-derived signals [182].

Downstream of PI-3'kinase.

Pleckstrin homology domains are structurally conserved modules of ~100 amino-acids that were first recognised in pleckstrin, a major phosphorylation substrate for PKC in platelets. Interestingly, the basic structure of PH-domains exhibits structural similarity to PTB-domains, a domain binding to phosphorylated tyrosine. PH-domains primarily bind to inositol lipids and their head groups although there are also examples of protein-protein interactions [183;184]. Several downstream effectors of PI-3'kinase signalling possesses PH-domains which selectively bind to 3'phosphorylated inositides. Three main classes of PH-domain containing signalling molecules acting downstream of PI-3'kinase have been described: the AGC-family of Ser/Thr protein kinases [185], the TEC-family of tyrosine kinases [186] and the Rho-family of GTPases (Fig. 2)[187].

The AGC-kinase family is a large family of Ser/Thr kinases archetyped by PKA, PKC and cGMP-dependent protein kinase. A major breakthrough in the PI-3'kinase dependent regulation of AGC-kinase members was the characterisation of 3'Phosphoinositide Dependent protein Kinase 1 (PDK1), capable of phosphorylating PKB on T^{308} in the presence of $PI(3,4,5)P_3$ (Fig. 2)[188-190]. PDKI posses a PH-domain capable of high-affinity binding to $PI(3,4,5)P_3$ [191], and possibly also with $PI(4,5)P_2$, localising this "master regulatory kinase" to the plasma membrane under basal conditions [188;192]. Aside from PKB, PDK1 can also phosphorylate several other AGC-kinase members on the activation loop Ser/Thr leading to full activation, such as S^{244} of PDK1 itself (in *trans*)[193] T^{229} of p70S6 kinase [194;195], T^{197} of cAMP-depend protein kinase [196] and T^{410} (/T^{403}) of PKC-ζ/λ [197].

PKB (also known as Akt) was originally identified as the oncogenic product transduced by the acute transforming retrovirus (Akt-8) isolated from an AKR-mouse thyoma [198]. In 1991 three independent research teams identified mammalian genes corresponding to PKB [199-201]. This important component of PI-3' kinase signalling provides a direct link between insulin-induced PI-3'kinase activity and a plethora of insulin actions such as glucose transport, glycogen and protein synthesis, gene expression and maintenance of cell viability (Fig. 2)[202-205].

The PKB family is conserved from Dictyostelium to man, but is not present in *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* suggesting PKB may have evolved coincidentally with the evolution of multicellular eukaryotic species.

PKBs are composed of : a C-terminal hydrophobic motif (HM)[206;207]. This HM-motif provides stability to the catalytic core of AGC-kinases by binding to a hydrophobic and phosphate-binding pocket, the so-called "PIF-pocket" [208-210]. A central catalytic domain similar to other

AGC-kinase family members [211]. And a PI(3,4)P_2- and PI(3,4,5)P_3-binding PH-domain at its N-terminus [212;213]. The association with these lipids however, does not directly lead to PKB activation *in vitro* [214]. Instead, PKB requires phosphorylation on two regulatory amino-acids T^{308}(in the activation loop of the kinase domain) and S^{473} (in the HM-motif) [215;216]. Whereas T^{308} phosphorylation is mediated by PDK1, the nature of S^{473} phosphorylation through a putative "PDK2" remains enigmatic. Stimulation of PDK1$^{-/-}$ ES-cells with IGF-1 results in a strong S^{473}-phosphorylation of PKB, ruling out an involvement of PDK1 or autophosphorylation [217]. Another candidate is integrin-linked kinase 1, which can phosphorylate S^{473} of PKB [218]. Furthermore interference with ILK1 results in loss of S^{473} phosphorylation [219;220]. However, ILK is a rather unusual kinase as it lacks several motifs deemed crucial in the kinase domain of other protein kinases (such as the Mg^{2+}-binding motif)[221]. Thus, rather than being the long-sought after PDK2, ILK may rather be an important scaffold or co-activator of this kinase-activity (Fig. 2).

PKB kinase exists as three different isoforms, α, β and γ. Of these PKB-β appears to be the main mediator of insulin signalling towards glucose uptake : During adipogenesis the levels of PKB-β increase, whereas the levels of PKB-α decline [222;223]. Insulin induced activation of PKB-β exceeds PKB-α activation in rat adipocytes [224]. Furthermore, micro-injection of antibodies or the application of siRNA against PKB-β (but not PKB-α) blocks insulin-induced GLUT4 translocation in 3T3-L1 adipocytes [222;225]. The most striking illustration is derived from the generation of isoform-specific knock-out mice. Mice lacking PKB-α demonstrate normal glucose homeostasis, but are small [226;227]. On the other hand, mice lacking PKB-β displayed insulin-resistance [227]. And indeed, adipocytes derived from these mice display an impaired insulin-induced GLUT4 translocation, which could be corrected after re-expression of PKB-β, but not PKB-α [228]. Thus, despite a high degree of sequence similarity, PKB-α is predominantly involved in control of growth/proliferation, whereas PKB-β regulates cellular metabolism.

An important mediator of insulin-signalling through PKB has been discovered in *C. elegans* [229-231]. When larvae of these worms are grown at high density, they enter the *dauer* stage, characterised by reduced metabolic activity, increased fat content and a doubling of life span [232]. Genetic mutants causing a constitutive *dauer* phenotype have been dubbed Daf-alleles (Dauer Arrest Phenotype). Remarkably, these include predominant members of the insulin-signalling pathway such as Daf-2 (IR/IGF-IR), Age-1 (PI-3'kinase) and the *C.elegans* orthologs of PKB [233-237]. Two mutants capable of suppressing this constitutive

phenotype are, Daf-16 and Daf-18 [238;239]. The former an orthologue of PTEN (which will be considered later), the second a member of the forkhead family of transcription factors (Fig. 2). In mammals, the family includes three expressed genes FKHR-L1, FKHR and AFX [240]. These genes are involved in transcriptional regulation of genes repressed by insulin [241-243]. Under basal conditions these forkheads reside in the nucleus and are able to initiate transcription. Following phosphorylation by PKB these transcription factors will be excluded from the nucleus and be retained in the cytoplasm [230;244].

PKB also phosphorylates and thereby activates phosphodiesterase 3B [245]. The activated phosphodiesterase hydrolyses cAMP and thereby down regulates the activity of PKA thus preventing the phosphorylation of perilipin and the activity of hormone-sensitive lipase [136].

Insulin-induced glycogen synthesis is catalysed by glycogen synthase. The constitutive active GSK-3β phosphorylates and inhibits glycogen synthase [246]. Phosphorylation of GSK-3β in turn by PKB generates a pseudosubstrate sequence which occupies the substrate-binding cleft of GSK-3β [247]. As a consequence, GSK-3β is inactivated thereby lifting the inhibition of glycogen synthase (Fig. 2). Important though this PKB target may be in most cell types, constitutive active PKB does not induce glycogen synthesis in 3T3-L1 adipocytes [248;249]. Surprisingly, this is due to the low expression of GSK-3β in 3T3-L1 adipocytes, in contrast to 3T3-L1 fibroblasts [248;250;251].

The identification of a protein kinase from rat brain activated by limited proteolysis lead to the identification of PKC [252;253]. The PKC-family consists of many different isoforms, subdivided in four separate classes on the basis of structural homologies and mechanisms of activation. All PKCs consist of an N-terminal pseudosubstrate domain, a regulatory domain and a C-terminal catalytic domain [254;255]. The pseudosubstrate domain is a sequence with the hallmarks of a PKC phosphorylation site, but has an alanine at the predicted Ser/Thr-phosphorylation site [256]. Consequently, this domain interacts with the catalytic domain and is responsible for intramolecular suppression of activity prior to effector binding. The conventional PKCs α, βI, βII and γ are further regulated by Ca^{2+} and phosphatidylserine-binding to their C2-domain and can be activated by the neutral lipid DAG or phorbol ester (PMA) binding to their C1-domain [257;258]. However, conventional PKC activity also depends on PDK1-mediated phosphorylation of the activation loop and subsequent autophosphorylation [197;259]. The splice variants PKC-βI and -βII differ only in a short C-terminal region of ~50 amino-acids, called the V5 region, which plays a critical role in differential subcellular targeting of these isoforms [260-262].

The novel PKCs ε, η, δ and θ are also sensitive to PMA, but lack one or more of the aspartate-residues required for Ca^{2+}-binding in their the regulatory C2-domain. Instead, their C2-like domains regulate PKC-activity through protein-protein interactions with RACKs [263-265]. The last two groups are first the atypical PKCs consisting of the isoforms λ (the human orthologue is called ι), ζ, the recently identified ζ II, and second the PKC Related Kinases 1-3 [254;266]. These kinases have only a partial, or no C1-domain and only a C2-like domain. The atypical PKCs can be activated by $PI(3,4,5)P_3$ whereas PRK bind to activated RhoA GTPase [267-271].

In insulin-signalling conventional and novel PKCs mainly act in a negative regulatory role (Fig. 2)[255;272;273]. The role of PKC-β in insulin-induced glucose uptake appears slightly more complex, on one hand it is involved in bypassing Ras during insulin-induced MAPK activation (Fig. 3)[274]. On the other hand PKC-β has been implied in directly phosphorylating and thereby negatively regulating the insulin-receptor[275-277]. Indeed, a PKC-β knock-out mouse demonstrates lowered blood glucose levels [278].

Of the atypical PKCs, 3T3-L1 adipocytes only express the λ-isoform [279]. The involvement of atypical PKCs downstream of PI-3' kinase is thoroughly characterised, such as by overexpression of either wild-type or dominant negative mutants, microinjection of PKC-λ antibodies or the application of pseudosubstrate peptides [267;279-283]. Most notable are the inhibition of atypical PKC by ASIP/PAR3 overexpression, which inhibits insulin-induced GLUT4 translocation and, loss of insulin-induced GLUT4 translocation in adipocytes derived from PKC-λ knock-out mice (Fig. 2)[284;285].

The TEC-family of tyrosine kinases are predominantly expressed in haematopoietic cells, with the notable exception of Etk [205]. Structurally they contain a C-terminal kinase domain and N-terminal SH2- and SH3 domains. Unlike the distantly related Src-kinase family, the TEC-family lacks a membrane-targeting myristoylation signal and an inhibitory Csk-targeted tyrosine-phosphorylation site. With the exception of Itk, all members contain an N-terminal PH-domain which binds $PI(3,4,5)P_3$ with high affinity *in vitro* [286]. And indeed PI-3'kinase activity is essential for TEC-kinase activation [287;288]. Once activated TEC-kinases can phosphorylate and activate PLCγ. The activity of PLCγ is further enhanced by direct association with $PI(3,4,5)P_3$ through it PH- and SH2-domains [289-291]. Active PLCγ subsequently hydrolyzes the relatively common phospholipid $PI(4,5)P_2$ to generate diacylglycerol and inositol 1,4,5-trisphosphate and hence mediates intracellular calcium release and c/nPKC activation [292]. This mechanistic cross talk between PI-

3'kinase- and PLCγ-signalling presents a straightforward hypothesis for the regulation of cPKC-activity in insulin signalling [293].
Guanine-nucleotide exchange factors convert small GTPases from the inactive GDP-bound form to the active GTP-bound form. Importantly, 3'phosphoinositide-binding PH-domains have been observed in all GEFs specific for the Rho family of GTPases (which includes Rho, Rac, Cdc42 and TC10)[294;295]. Strikingly, these GTPases have been implicated in regulation of the actin cytoskeleton and in vesicular trafficking, cell-morphological processes known to be intimately linked to insulin-induced glucose uptake (Fig. 2). In rat adipocytes Rho induces the activity of PC-PLD via PI-3'kinase signalling, leading to another potential mechanism for insulin induced activation of DAG-regulated PKCs [296-301].

The CAP-Cbl axis of insulin signalling.
A recent breakthrough has been made by the identification of insulin-induced Cbl-tyrosine phosphorylation in 3T3-L1 adipocytes (Fig. 5)[302]. Once phosphorylated, Cbl functions as a scaffold, associating with the adapter protein Crk II, the tyrosine-kinase Fyn [302] and the adapter protein CAP [303]. CAP consists of an N-terminal Sorbin Homology domain followed by three SH3 domains at the C-terminus, with constitutive Cbl-association mediated by the most C-terminal SH-3 domain [303;304]. Upon insulin stimulation, the CAP-Cbl complex transiently associates with the Insulin Receptor mediated by the adapter protein APS [305]. APS is a member of the Lnk family of adapter proteins that is highly expressed in insulin-responsive tissues such as fat, skeletal muscle and heart [306]. Upon receptor activation APS-dimers engage two phosphotyrosines in the activation loop of the Insulin Receptor (Y^{1158} and Y^{1162}) through their SH2-domains [307]. Subsequent tyrosine phosphorylation APS on Y^{618} induces a binding site for the Tyrosine Kinase Binding-domain of Cbl [305].
The Cbl-family are the cellular homologues of the transforming v-Cbl oncogene [308;309]. This family of scaffolds comprises of c-Cbl, Cbl-b and Cbl-c [310]. Apart from their N-terminal TKB domain, Cbl consists of a RING finger domain, multiple proline-rich stretches, several potential tyrosine phosphorylation sites and a conserved ubiquitin-associated domain. APS facilitated tyrosine phosphorylation of Cbl (on Y^{371}, Y^{700} and Y^{774}) by the Insulin Receptor induces the APS-CAP-Cbl complex to translocate to the caveolae (Fig. 5)[305;311;312]. This translocation is mediated through the association of the SoHo-domain of CAP with the caveolar residential protein flotillin [304;313].

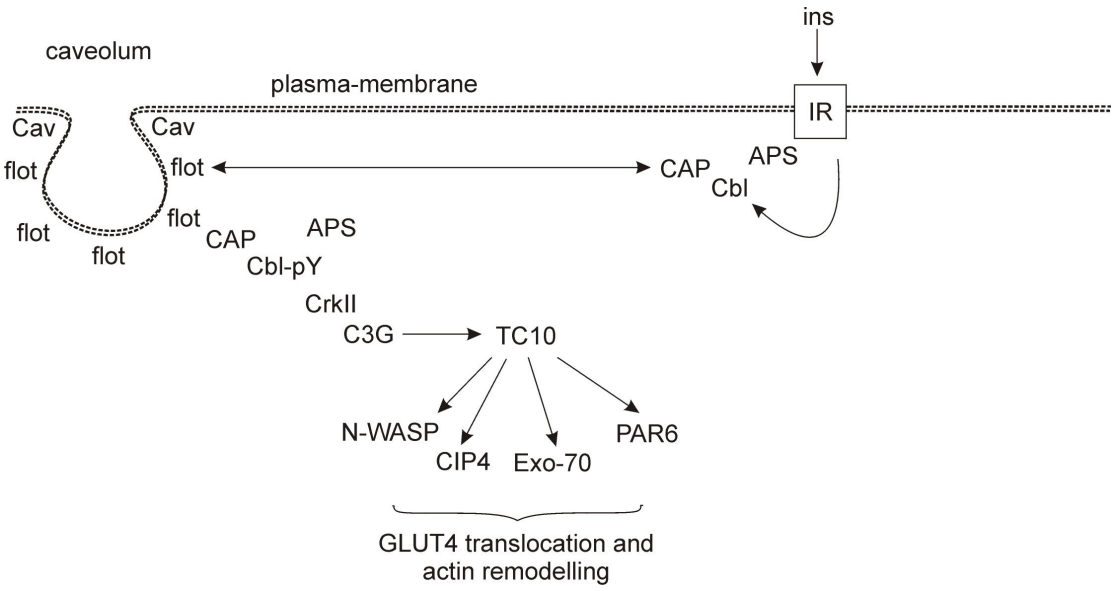

Fig. 5 Cbl signalling routes
Insulin-induced signalling routes in 3T3-L1 adipocytes. Activation steps are indicated by arrows. After activation of the APS-CAP-Cbl complex by the insulin receptor the whole complex moves to the caveolum (see the next chapter for more information on this adipocyte cell-morphological structure). The full name of all protein components can be found in the list of abbreviations at the end of this thesis.

In the caveolae, Cbl-associated CrkII binds C3G, which functions as an exchange factor for the caveolar residential small G-protein TC10 [314]. Both isoforms of TC10 (α and β) are activated in response to insulin, however, only ectopic overexpression of TC10α disrupts cortical actin and inhibits insulin-induced GLUT4 translocation [315]. Active GTP-bound TC10 can bind a number of potential effectors, including mixed lineage kinase 2, myotonic dystrophy related Cdc42 kinase, p21 activated protein kinases, the Borg-family of interacting proteins, the mammalian partition defective homologue Par6, the microtubule-interacting protein CIP4, the N-WASP isoform of the Wiskott-Aldrich syndrome Protein and Exo70 of the Exocyst complex [316-321]. Concomitantly TC10 also mediates extensive cortical actin depolymerisation and increased perinuclear actin polymerisation (Fig. 5)[322].
Remarkably however, an APS knock-out mouse displays increased insulin sensitivity [323]. And, APS knock-out does not affect insulin induced GLUT4 translocation in adipocytes derived from these mice [323]. It seems likely other Lnk-family members (such as SH2-B) are capable of mediating these responses in an APS-null background [324].

MAPK-signalling

This pathway is largely under control of RasGTP formation in response to insulin [325-328]. All MAPK pathways include central three-tiered signalling modules in which MAPKs are activated by concomitant Tyr and Thr phosphorylation. This dual phosphorylation is mediated by a family of dual specificity kinases referred to as MAPK/Extracellular signal regulated Kinases (MEK) which are themselves subject to regulatory Ser/Thr phosphorylation (Fig. 3)[329;330]. Though MAPKs are proline-directed Ser/Thr kinases, all substrates also contain specific MAPK docking-sites, conferring specificity on the signalling capacity of the different MAPK subfamilies [331-335]. Furthermore, scaffold proteins bind and select specific MAPK components, conferring an additional layer of signalling specificity on the MAPK-pathways [53].

The p38 MAPKs were originally identified as cellular stress-induced protein kinases [336;337], although p38 MAPK is also activated by some hormones and growth factors [338]. p38 MAPKs are activated by dual phosphorylation on their activation loop, T^{180} and Y^{182} in a TGY tripeptide motif [339]. At least four isoforms, p38α, p38β, p38γ and p38δ, and two splice variants, p38α2/Mxi2 and p38β2 have been described [340-349]. The p38 MAPK isoforms differ in expression, substrate preference and sensitivity to SB203580 (with only the α- and the β-isoforms affected by this pharmacological inhibitor)[340;350-352]. Of these isoforms, insulin induces the activation of p38α and p38β MAPK in 3T3-L1 adipocytes and L6-myotubes, but not in 3T3-L1 fibroblasts or L6-myoblasts [353;354]. Interestingly, SB203580 reduced insulin-induced glucose uptake in 3T3-L1 adipocytes and L6 muscle cells without affecting GLUT4 translocation towards the plasma-membrane [355]. Furthermore, expression of an inducible dominant-negative p38 MAPK mutant similarly affected glucose uptake without interfering with GLUT4 translocation [356]. Thus p38 MAPK activation by insulin alters the relative speed of glucose transport (Fig. 3).

Apart from its contribution in insulin-induced glucose uptake, prolonged p38 MAPK signalling impedes insulin-signalling pathways through the phosphorylation of IRS-1 and a down regulation of GLUT4 levels (Fig. 3)[357-360]. Indeed, in adipocytes and skeletal muscle of type II diabetic patients a loss of insulin-induced p38 MAPK phosphorylation with a concomitant increase in basal p38 MAPK phosphorylation has been reported [361;362]. Thus aberrant p38 MAPK signalling might contribute to the pathogenesis of insulin resistance.

Downstream of p38 MAPK, MAPKAP-K2 is a member of a small family of Ser/Thr kinases consisting of an N-terminal regulatory domain and a C-terminal catalytic domain [363]. MAPKAP-K2 is phosphorylated and

activated by p38α and p38β (but not by p38γ or p38δ)[337;364;365]. Along with another p38 MAPK substrate kinase, PRAK [366], MAPKAP-K2 phosphorylates the small heat shock protein HSP-27 (Fig. 3). Phosphorylation coincides with relocalisation of HSP-27 to the actin cytoskeleton were it affects the organisation of F-actin [367-369]. Directly upstream of p38 MAPK are the dual-specificity MAPK kinases MKK3 and MKK6, with a possible involvement of auto-phosphorylation as well [370-372]. However, it is unclear if MKK-3/6 phosphorylation is involved in insulin induced p38 MAPK activation in adipocytes (Indicated with a question mark in Fig. 3).
Alternatively, the PAK family of Ser/Thr kinases are structural and functional mammalian orthologs of *S. cerevisiae* Ste20p [373]. PAKs plays a critical role in mediating cytoskeletal organisation and regulation. Indeed, PAK-1 has been shown to translocate into cortical actin structures after stimulation with insulin [374]. PAK1 binds and is activated by Rac1-3 [375-377], Cdc42 [375] and TC10 [316]. Via their N-terminal PxxP motifs PAKs can also interact with SH3-domain containing adaptor proteins enabling recruitment to tyrosine kinases [378-381]. Furthermore, both PKB and PDK1 have been implicated as upstream regulatory kinases [382-384]. Several reports indicate that PAKs (in analogy to their yeast orthologue) can activate p38 MAPK [385-388].
Aside from its metabolic effects, insulin also stimulates the MAP kinases ERK-1, -2 through MEK-1 and –2 [329]. In adipocytes, introduction of IRS-1 antisense RNA, antibodies to IRS-1 or a point mutation in the Grb-2 binding site on IRS-1 attenuate the effect of insulin on ERK-signalling and concomitant DNA-synthesis (Fig. 3)[389-391]. Insulin-stimulation induces the association of Grb-2 with IRS-1. In turn the adaptor protein Grb-2 recruits the Son-of-sevenless exchange protein for the activation of Ras inducing the conversion of Ras from a GDP-bound to an active GTP-bound form [392;393]. Aside from IRS, Grb2 also binds to phosphorylated Y^{317} of Shc, suggesting that this insulin receptor substrate is also involved in mediating mitogenic signalling (Fig. 3)[394]. In many continuous cell culture systems Shc is believed to be the major Grb2/Sos activator during insulin stimulation [389;395]. Conversely however, in skeletal muscle IRS-1 is the predominant Grb-2 binding protein, whereas Grb-2 binds poorly to Shc in this background. Thus IRS-1 knockout mice display an 80% reduction in insulin-stimulated Ras activation [396]. Indeed, Shc phosphorylation is disconnected from insulin-signalling during 3T3-L1 adipogenesis [397;398]. And in subcellular fractionation-assays of adipocytes insulin-stimulated phosphorylation of Shc occurs exclusively in the plasma-membrane, whereas mSOS was only observed in the IRS-1 containing LDM-fraction [119].

Once activated, the "conventional" pathway dictates Ras functions as a molecular switch stimulating a stepwise activation of Raf, MEK and ERK. Activated ERK can then translocate to the nucleus, where it catalyses the phosphorylation of transcription and translation factors such as SAP, PHAS-I and Elk initiating a cellular programme that leads to cellular proliferation or differentiation [399-404].
However, terminally differentiated 3T3-L1 adipocytes present a twist to this tale, once more illustrating how the wiring of signalling pathways can be tuned cell-type specific to suit its own unique requirements in a given cellular environment. In 3T3-L1 adipocytes, insulin-induced activation of ERK-1/-2 is disconnected from the insulin-induced Ras-Raf pathway (Fig. 3)[405-410]. The insulin-induced ERK-phosphorylation is mediated through PKC-signalling bypassing the Ras-Raf axis of signalling [274;411]. Concomitantly, PKB has been shown to inhibit Raf protein kinase through S^{259} phosphorylation and subsequent 14-3-3 association [412]. This inhibition of Raf by PKB does not operate in undifferentiated myoblast precursor cells, but does when these cells are differentiated into skeletal-muscle myotubes [413]. (see also Rao for a review on similar differences in MAPK-signalling between primary cells and established continuous cell lines [414]). The precise reason for this differential signalling is unclear. At any rate, it has been unambiguously demonstrated that this mitogenic-signalling cascade does not play a role in mediating the metabolic effects of insulin [415-423].

Phosphatases and insulin-induced signalling pathways
As in every signalling system, an elaborate mechanism of phosphatases exists to ensure rapid termination of the insulin-induced signalling cascade and to keep the signalling pathways silent in the absence of insulin. Consequently, aberrant regulation of phosphatases results in an inability for the insulin-signalling pathway to activate glucose uptake. Aside from their role in "resetting" the system back to the basal state when the insulin-stimulus has ended, some phosphatases apparently play a positive stimulatory role in insulin-signalling. Most notable are the Ser/Thr phosphatase PP1 and the tyrosine phosphatase SHP2 (Fig. 2 and 3). The activation of Ras requires the tyrosine phosphatase SHP-2, through its interaction with IRS-1/-2 [424-426]. Although the precise mechanism is poorly understood, ectopic overexpression of inactive SHP-2 mutants attenuates insulin-induced Ras activation [425;427]. Several studies have revealed PTPs that are active against the autophosphorylated insulin receptor, including the receptor-like CD45, leukocyte antigen-related PTP (LAR) and the cytosolic PTP-1B (Fig. 1)[428-433].
The tyrosine-phosphatase LAR has often been implied as a key-regulator of insulin receptor activity [434] and LAR-deficient mice exhibit

profound defects in glucose homeostasis [435]. Similarly, another tyrosine-phosphatase PTP-1B also regulates the insulin receptor tyrosine-kinase. Ablation of PTP-1B in mice presents an insulin-sensitivity syndrome, as well as resistance to diet-induced insulin-resistance [436-438]. Expression and activity of PTP-1B are tightly regulated by $G\alpha_{i2}$-signalling mediated through the PKA-pathway (Fig. 1). Thus, transgenic mice with a targeted expression of the GTPase deficient, constitutively active $Q^{205}L$ $G\alpha_{i2}$-mutant results in significantly improved insulin sensitivity [439]. Conversely G_s (leading to enhanced cAMP and consequently PKA activity) negatively regulates insulin signalling, possibly through the same pathway [440].

With respect to the phosphoinositides, at least two groups of lipid phosphatases have been described. Members of the first group remove the D5 phosphate from the inositol ring [441-443] and carry an N-terminal SH2 domain. Members of this group include $p150^{SHIP}$/SIP-130 [444], its splice variant SIP-110 which lacks the SH2-domain [445;446], SHIP2 [447;448] and INPPL1 (Fig. 2)[449;450].

This class of lipid phosphatases removes the 5'phosphate of $PI(3,4,5)P_3$ and as such they form the prime source of $PI(3,4)P_2$ in cells [441]. Intriguingly, TAPP-1, an $PI(3,4)P_2$ adapter protein mediates the translocation of PTP-L1, a tyrosine-phosphatase, towards the plasma-membrane (Fig. 1). Consequently, this adapter-phosphatase complex may be an important factor in terminating the insulin signal after the degradation of $PI(3,4,5)P_3$ to $PI(3,4)P_2$ [451]. Ectopic overexpression of either SHIP-1 or SHIP-2 in 3T3-L1 adipocytes results in a loss of insulin induced PKB activation suggesting the need for $PI(3,4,5)P_3$ in mediating these responses to insulin *in vivo* [448;452;453].

The second group of lipid phosphatases is represented by PTEN [454;455], which targets the D3'-phosphate [456]. The identification of a PTEN-mutation in Cowden's disease [457] as well as its inhibitory effects on PKB activation [458;459] illustrate its prime importance as a lipid phosphatase antagonising PI-3'kinase signalling (Fig. 2). Ectopic expression of PTEN hampers insulin-induced glucose uptake in 3T3-L1 adipocytes [460;461].

In 3T3-L1 adipocytes, overexpression of constitutively active PKB does not induce glycogen synthesis [248]. Rather than PKB/GSK-3β it is the insulin-induced activation of a phosphatase (PP1) in 3T3-L1 adipocytes (but not in 3T3-L1 fibroblasts) that dephosphorylates and activates glycogen synthetase [250]. Though PKB is not involved, PI-3'kinase activity is, as the insulin induced activation of PP1 is inhibited by wortmannin (Fig. 2)[250]. PP1 is a cytosolic protein phosphatase which is compartmentalised in cells by discrete targeting subunits. The predominant PPI glycogen targeting subunit in 3T3-L1 adipocytes is PTG

[462]. PTG functions as a molecular scaffold, binding not only to Glycogen Synthetase, but also to Phosphorylase and Phosphorylase kinase. Consequently, aside from activating glycogen synthetase, PPI-activity concomitantly inhibits glucogenolysis, contributing to the storage of glucose in the glycogen particle [463;464].
PP2A is a multimeric Ser/Thr phosphatase that has been highly conserved during the evolution of eukaryotes. In mammals, the core enzyme is a dimer, consisting of a catalytic ($PP2A_C$) and a tightly associated regulatory subunit termed PR65 or A subunit. Two distinct isoforms exist of both the catalytic and regulatory subunits [465]. A knock-out of $PP2A_{C\alpha}$ is not viable, demonstrating that although highly homologues, these isoforms play non-redundant roles *in vivo* [466]. Although the presence of this core structure has been observed *in vivo* prevalent PP2A enzymes are heterotrimers through the association with another regulatory subunit. These B-subunits form a large family of proteins (classified as B, B', B'' and B''') each consisting of several isoforms, resulting in a grand total of about 75 different PP2A enzymes. The B-subunits demonstrate a very specific subcellular localisation, developmental regulation and cell-type specificity thus tightly and precisely regulating the activity of PP2A. Aside from association with specific B-subunits, PP2a may be further regulated through covalent modification. PP2A has mainly been implicated as an important negative regulator of AGC-kinases, the ERK-family and PAK [467-469]. Thus, osmotic shock directly inhibits insulin-induced PKB activity by activating a specific PP2A-like phosphatase (Fig. 2)[470;471]. Furthermore, PP2A forms a molecular complex with Shc, thereby negatively regulating the Ras/MAP kinase pathway emanating from Shc (Fig. 3)[472].
Apart from PP2A, another important regulator of the MAPK-family is the MKP-family of dual-specificity phosphatases, which are able to dephosphorylate MAP kinases on both serine/threonine- and tyrosine-residues simultaneously. Several layers of regulation confer specificity on this family of phosphatases, including differential transcription in response to external stimuli and cell-type specific expression patterns [473;474]. Furthermore, whereas most MKPs reside in the nucleus, MKP-3, -4,-7 and (dependent on cellular environment) hVH-5 are cytosolic [475-479]. In general there is a good correlation between docking ability and the activity of MKPs towards MAPKs [480-486]. Thus, MKP-3 and hVH-5 exhibit highly selective binding and subsequent inactivation of either ERK or JNK and p38 MAPK respectively [477;478]. However, while PAC-1 inactivates JNK in COS7 cells, it could not act effectively on JNK in either NIH3T3 or HeLa cells [487]. Furthermore, in EGF-treated fibroblasts, MKP-1 provides the main phosphatase activity for ERK inactivation. By contrast, in EGF-treated adipocytes, MKP-1 is

dispensable and PP2A is the main phosphatase mediating ERK inactivation (Fig. 3)[488]. Thus although MKP-1 has been originally identified as a ERK-1/2 phosphatase [489], it is the upregulation of this dual-specificity phosphatase after dexamethasone-treatment and concomitant dephosphorylation of p38 MAPK activity that has spawned considerable interest in this protein [490-494]. Another MKP-family member recently implicated in the pathogenesis of type II diabetes is MKP-4. This dual-specificity phosphatase is localised in the cytoplasm of cells and is also capable of dephosphorylating p38 MAPK (Fig. 3)[476;495]. Intriguingly, MKP-4 is upregulated in adipocytes derived from *ob/ob* and *db/db* mice [496].

The GLUT-transporters

More than half a century ago Levine *et al* described insulin-induced glucose uptake [497], though at the time this was suggested to be mediated by an increase in membrane permeability and/or fluidity. Decades thereafter two seminal papers illustrated that glucose uptake occurs through the insulin-induced translocation of facilitative glucose transporters [498;499]. Currently there are 13 members of this family of facilitative glucose transporters, GLUT1-12 and the myo-inositol transporter HMIT1, each with different tissue distributions, kinetic properties and sugar specificity [500-502]. Best characterised are GLUT1-4, forming a subgroup within this family called class I glucose transporters. Of these, GLUT1 is ubiquitously expressed and responsible for basal levels of glucose uptake in all tissues. The GLUT2 isoform is primarily expressed in the beta-cells and in the liver. It has a relatively high Km (app) for glucose and serves as part of a glucose sensor in these cells and mediates absorption of glucose by intestinal epithelial cells. GLUT3 has the highest affinity for glucose and is expressed in neurons and during foetal development. The GLUT4 isoform is predominantly restricted to adipose and muscle tissue where it is sequestered in intracellular vesicular structures. Upon insulin stimulation these vesicles translocate and fuse with the plasma-membrane thereby causing an increase in the number of available transporters mediating the effects of insulin on glucose uptake in these cells.

Two models have been proposed for the mechanism GLUT4 vesicle translocation in response to insulin : a retention model and a docking/fusion model [503;504], which do not have to be mutually exclusive. The latter predicts insulin-induced GLUT4 vesicle fusion occurs through the specialised docking proteins called SNAREs (Fig. 6). VAMP-2 is the main v-SNARE (v for vesicle) found in GLUT4 vesicles [505;506]. The main t-SNAREs (t for target-membrane) found in adipocytes are syntaxin-4 and SNAP-23 [507-510].

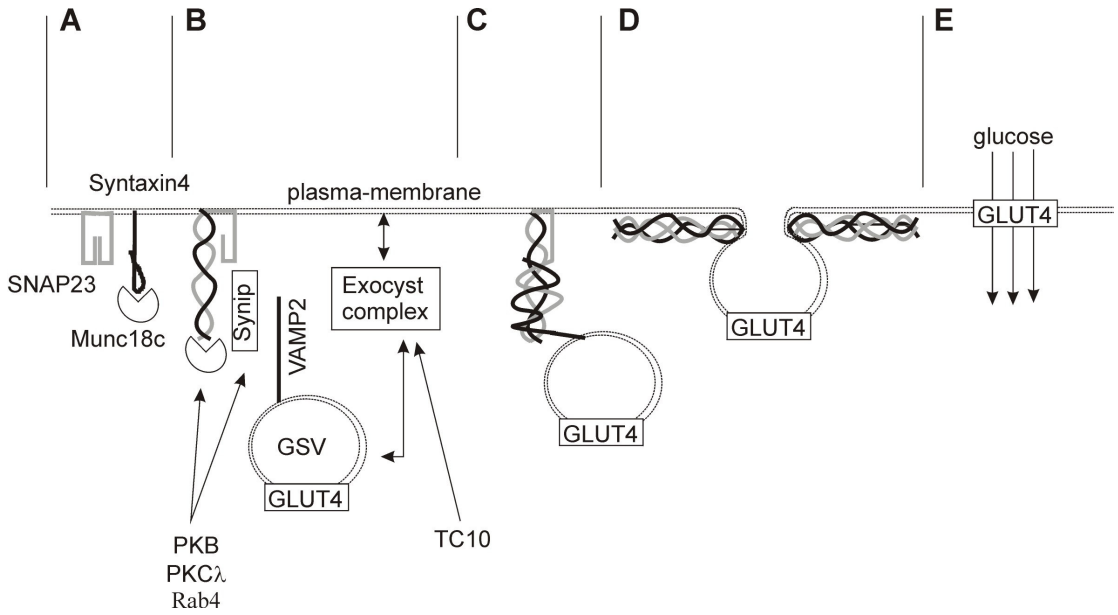

Fig. 6 v/t-SNARE signalling routes
Insulin-induced vesicle (v)- and target (t)- SNARE vesicle fusion routes in 3T3-L1 adipocytes. A. SNAP-23 and syntaxin-4 are tethered to the plasma-membrane. The syntaxin associating protein Munc18c is involved in "priming" the syntaxin and allowing coiled-coil formation with SNAP-23. B. The primed complex is stabilised by the activities of Munc18c and Synip inhibiting further fusion. Rab4, PKB and PKC-λ activities leads to dissociation of these proteins from the t-SNARE complex. These activation steps are indicated by arrows. The v-SNARE containing GLUT4 Storage Vesicle is tethered to the plasma-membrane by the activity of the Exocyst complex under the control of TC10, indicated by double arrows. C. Subsequently the trans-conformation is formed. D. Zippering up of the v/t-SNARE complexes in a coiled-coil complex provides the energy required to induce fusion of the vesicular- and plasma-membranes. In A-C only one complex is shown for clarity, though in the cell numerous complexes circumventing the site of fusion are present. In D. this is depicted by the presence of coiled-coils on both sites of the membrane "neck". E. The v/t-SNARE complexes dissociate and are recycled. The fully embedded GLUT4 glucose transporter is now available for the uptake of glucose from the extracellular milieu. the full name of all protein components can be found in the list of abbreviations at the end of this thesis.

Insulin-stimulated GLUT4 translocation is dependent upon the interaction of VAMP-2 with syntaxin-4 and SNAP-23 at the plasma-membrane [510;511]. With SNAP23 mediating the interaction between the former two [512]. VAMP2 has been described as a target of both PKB-β and PKC-λ, which could provide a direct link between the PI-3' kinase pathway and vesicle-fusion machinery [513;514].
All syntaxins are transmembrane proteins anchored by their C-terminal domains and the rest facing the cytoplasm. Several cytosolic but

hydrophobic regions, called the SNARE domain, have the potential to form coiled-coil α-helical structures (Fig. 6)[515;516].
The SNARE domain of syntaxin mediates its interactions with the SNARE domain of other t-SNARES of the SNAP-family, such as SNAP23, in turn this complex can associate with a v-SNARE, such as VAMP2. Consequently an extremely (heat- and SDS-resistant) stable ternary complex formed by a twisted bundle of α-helices spanning roughly 12 nm is formed [517-519]. In the initial stage of vesicle docking, the SNARE complex assumes a partial and reversible assembly known as the "*trans*-conformation" (Fig. 6C). In this case syntaxin is slightly less tightly associated with the VAMP and SNARE until a signal stimulates the zippering up of the complex bringing the membranes in close vicinity and concomitantly providing the free energy needed for membrane fusion (Fig. 6D)[520].
The retention model predicts that rather than active transport of the vesicle, the GLUT4 vesicle exists partly in this pre-docked "*trans*-conformational" state with the inhibitory activity of several accessory proteins being alleviated by insulin leading to full vesicle fusion (Fig. 6B). Several insulin-dependent syntaxin-4 binding proteins capable of regulating vesicle fusion have been described such as Synip [521] and Munc-18c [522-524]. Structural analysis demonstrated that Munc-18 plays a double-role in regulating syntaxins, on one hand it blocks vesicle fusion, presumably through direct steric interference by its association with syntaxin. Conversely however, Munc-18c has also been implicated in priming syntaxin for subsequent SNAP and VAMP association by changing the conformation of syntaxin into a semi-open structure. Munc-18c, regulated by the Rab GTPases in conjunction with the actin cytoskeleton has been shown to specifically modulate insulin-stimulated GLUT4 translocation [522;524;525]. Furthermore, O-linked glycosylation of Munc-18c has been implicated in glucosamine-induced insulin resistance [526].
The yeast Exocyst complex consists of eight proteins : Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84, and are involved in the tethering or docking of exocytotic vesicles [527]. The Exocyst complex assembles at the plasma membrane of adipocytes in response to insulin, through the association of Exo70 with the aforementioned TC10 [317]. By tethering the GLUT4 vesicle in the vicinity of the t-SNAREs this complex regulates GLUT4-vesicle fusion with the plasma-membrane (Fig 6B)[509]. Importantly, ectopic overexpression of an N-terminal fragment of Exo70 blocks GLUT4-vesicle membrane fusion, rather than GLUT4-vesicle translocation reminiscent of the effect of described APS-mutants [305;317].

In conclusion, the ability to elicit specific biological responses when stimulated with a given hormone is a remarkable feat of cells. This becomes even more remarkable when realising that many signalling pathways employ common components. Over the past years, analysis of insulin signalling pathways in cell-types such as adipocytes and muscle cells has yielded insight into how the insulin signalling pathways are routed and regulated cell-type specifically in time and space. Consequentially, an adipocyte is able to respond specifically to the presence of insulin with an increase in glucose uptake from the extracellular environment in a matter of mere minutes.

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