Critical nodes in signalling pathways: insights into insulin action

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Abstract | Physiologically important cell-signalling networks are complex, and contain several points of regulation, signal divergence and crosstalk with other signalling cascades. Here, we use the concept of 'critical nodes' to define the important junctions in these pathways and illustrate their unique role using insulin signalling as a model system.

Critical node
A point in a signalling network that is essential for the biological function of a ligand–receptor interaction, but also allows divergence of the signal to facilitate crosstalk between systems and/or to fine-tune the response to stimuli.

Insulin receptor substrate protein
(IRS protein). A large protein scaffold that serves as a docking platform for other signalling proteins that contain Src-homology-2 domains. The IRS proteins are required for a complete insulin signal.

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Signalling by membrane receptors is essential for the regulation of several cell functions, but how each ligand–receptor interaction produces its own specific pattern of regulated cellular function is a fundamental question in biology. The identification of specific receptors and second messenger systems, such as cyclic AMP (cAMP), led to the classical view of signalling as a linear cascade (FIG. 1a). Over time, this linear cascade evolved to reflect the divergence that is observed at several steps in a signalling pathway and the crosstalk between signalling pathways (FIG. 1b,c). However, it is now apparent that even this model might not be sufficient to explain the complexity of cellular signalling and the integrated control of cellular functions.

One of the most daunting challenges to understanding any particular ligand–receptor system is the identification of components, or nodes, within the network that are essential mediators or modifiers of the ligand's signal[125]. Several groups have taken quantitative approaches that integrate biochemical and computational data to identify essential nodes in signalling networks, as well as to describe how these nodes might interact with other signalling cascades. We believe that a complementary approach to describing cell-signalling networks, using data that has been derived from *in vitro* and *in vivo* genetic knockout studies, could provide a useful framework for integrating the growing body of knowledge in the field.

Here, we propose that the essential mediators, or 'critical nodes', of any signalling network (FIG. 1d) can be identified by three criteria: first, the node must constitute a group of related proteins (for example, gene isoforms) that are essential for the receptor-mediated signal, and in which two or more of these related proteins might have unique biological roles within a signalling network and therefore serve as a source of divergence within the signalling system; second, the node is highly regulated, both positively and negatively; and third, the node is a junction for potential crosstalk with other signalling systems. We illustrate this concept by analysis of the insulin-signalling network, in which several critical nodes have been defined and shown to have important roles in normal physiology, and disease states such as diabetes, obesity and the metabolic syndrome.

Complexity of the insulin-signalling network

Insulin signalling is mediated by a complex, highly integrated network that controls several processes. In the presence of insulin, the insulin receptor (IR) phosphorylates insulin receptor substrate proteins (IRS proteins) that are linked to the activation of two main signalling pathways: the phosphatidylinositol 3-kinase (PI3K)–AKT/protein kinase B (PKB) pathway, which is responsible for most of the metabolic actions of insulin, and the Ras–mitogen-activated protein kinase (MAPK) pathway, which regulates expression of some genes and cooperates with the PI3K pathway to control cell growth and differentiation (reviewed in REF. 1) (FIG. 2).

Although a simple scheme of divergent pathways looks sufficient to explain insulin signalling, the concept of critical nodes becomes apparent when one looks at the sheer number of gene and protein isoforms that are involved in the activation of AKT/PKB and the generation of metabolic effects. For instance, the IR has two splice isoforms, which are usually co-expressed in cells that also express the highly related insulin-growth factor-1 receptor (IGF1R) that can also be activated by insulin. Both the IR and IGF1R can phosphorylate at least six known substrate proteins that are capable of interacting with eight known forms of the PI3K regulatory subunit, which associate with three forms of the PI3K catalytic subunit that can generate phosphatidylinositol-3,4,5-triphosphate (PIP_3), which leads to the activation of the three known isoforms of AKT/PKB. Therefore, when you consider the combinatorial possibilities in these first steps of the pathway, there are over 1,000 possible combinations, and this number increases further

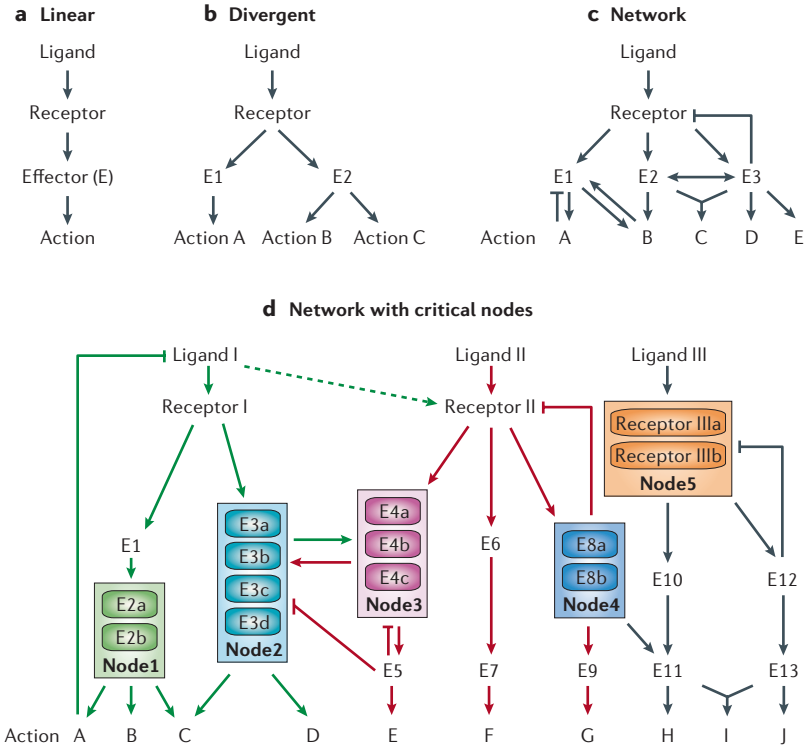

Figure 1 | **Evolution of our concepts on signalling pathways.** The binding of a ligand to its receptor triggers the activation of signalling pathways through effector (E) proteins that transduce signals to several intracellular second-messenger systems, which eventually lead to biological actions. Owing to the several molecules that are involved in these processes, our concepts of signalling pathways have evolved from simple linear cascades (**a**) to complex networks (**b** and **c**), and currently networks with critical nodes that participate in the crosstalk between the signalling networks (**d**, represented by the shaded/coloured boxes). In panel **d**, network 1 is represented by the green arrows, network 2 by the red arrows, and network 3 by the black arrows. Plain arrows represent an activation process, dashed arrows represent an activation process with less intensity and blocked arrows represent an inhibition process. The numbers beside every component of the network (I, II, III for receptors and 1 to 13 for effectors) represent distinct proteins, whereas the small letters indicate different isoforms of the same protein.

if we consider the compartmentalization, kinetics and downstream components of the signalling network.

Among the hundreds of molecules that have been shown to be involved in the insulin-signalling pathway, we have identified the IR/IRS, the PI3K heterodimer and AKT/PKB (FIG. 2) as the three best-defined critical nodes. As we will explain, each of these network components constitutes a critical node because they satisfy the criteria discussed above — each is essential for insulin action, consists of several isoforms with unique functions, is highly regulated and also mediates crosstalk with other signalling cascades. Most importantly, these critical nodes have been tested extensively as essential mediators of insulin signalling, both *in vitro* and *in vivo*. Other critical nodes in the insulin-signalling network might exist, however, only the IR/IRS, PI3K and AKT/PKB nodes have the strength and preponderance of data at this time to be considered 'critical'. Finally, we will speculate on the existence of other possible critical nodes, and discuss approaches to investigate these further.

Receptor tyrosine kinase (RTK). A cell-surface receptor with an intracellular tyrosine kinase domain. The ligand-mediated activation of an RTK results in the activation of the intracellular kinase domain.

Insulin resistance
Insulin resistance is a condition in which normal concentrations of insulin produce a subnormal biological response. It is common in many physiological and pathological states, including obesity, type-2 diabetes, metabolic syndrome, polycystic ovarian disease, pregnancy and puberty.

The IR and the associated IRS node

The first critical node in the insulin-signalling network is, by definition, the IR and the associated IRS proteins (FIG. 2). It is important to note that unlike other receptor tyrosine kinases (RTKs) that bind directly to the cytoplasmic tails of downstream effectors[2], the IR and IGF1R satellite proteins that are known as the IRS proteins mediate the binding of intracellular effectors. In addition, the IR and the IRS proteins share common mechanisms of regulation: they are activated by tyrosine phosphorylation, and they are negatively regulated by protein tyrosine phosphatases (PTPs), serine phosphorylation and ligand-induced downregulation.

The activity of the IR is tightly regulated, as unchecked activation or inactivity would lead to profound metabolic consequences. There are two splice isoforms of the IR, and each has a different affinity for insulin and IGF1 (see text, BOX 1). In addition, there are several mechanisms of negative regulation. One class of regulatory proteins is tyrosine phosphatases, the most studied of which is PTP1B. PTP1B interacts directly with the IR and dephosphorylates important tyrosine residues, thereby reducing its activity. Knockouts of PTP1B have shown remarkable efficacy in improving insulin sensitivity *in vivo* through enhanced insulin signalling[3]. Other proteins, such as suppressor of cytokine signalling-1 (SOCS1) and SOCS3 (REF. 4), growth-factor-receptor-bound protein 10 (Grb10) and plasma-cell-membrane glycoprotein-1 (PC1) downregulate IR function by sterically blocking its interaction with the IRS proteins, or by modifying its kinase activity. The SOCS proteins are of particular importance because they have been shown to be upregulated in states of insulin resistance, such as obesity, and therefore might contribute to the pathophysiology of diabetes[5]. The IR is also downregulated at the protein level by ligand-stimulated internalization and degradation, which is a common feature of most insulin-resistant, hyperinsulinaemic states, including obesity and type-2 diabetes[6].

Insulin-receptor substrates. At least 11 intracellular substrates of the IR and IGF1R kinases have been identified. Six of these belong to the family of IRS proteins, and have been termed IRS1–6 (REFS 7–11). Other substrates of the IR/IGF1R include Grb2-associated binder-1 (Gab1)[12], Cas-Br-M (murine) ecotropic retroviral transforming sequence homologue (Cbl)[13] and the various isoforms of Src-homology-2-containing protein (Shc)[14]. IRS1 and IRS2 are widely distributed, whereas IRS3 is largely limited to the adipocytes and brain, and IRS4 is expressed primarily in embryonic tissues or cell lines. IRS5 and IRS6 seem to have limited tissue expression, and function in signalling[11].

The IRS proteins have both pleckstrin-homology domains (PH domains) and phosphotyrosine-binding domains (PTB domains) near the N terminus that account for the high affinity of these substrates for the IR (FIG. 3). The centre and C terminus of the IRS proteins contain up to 20 potential tyrosine-phosphorylation sites that, after phosphorylation by the IR, bind to intracellular molecules that contain Src-homology-2 domains (SH2 domains).

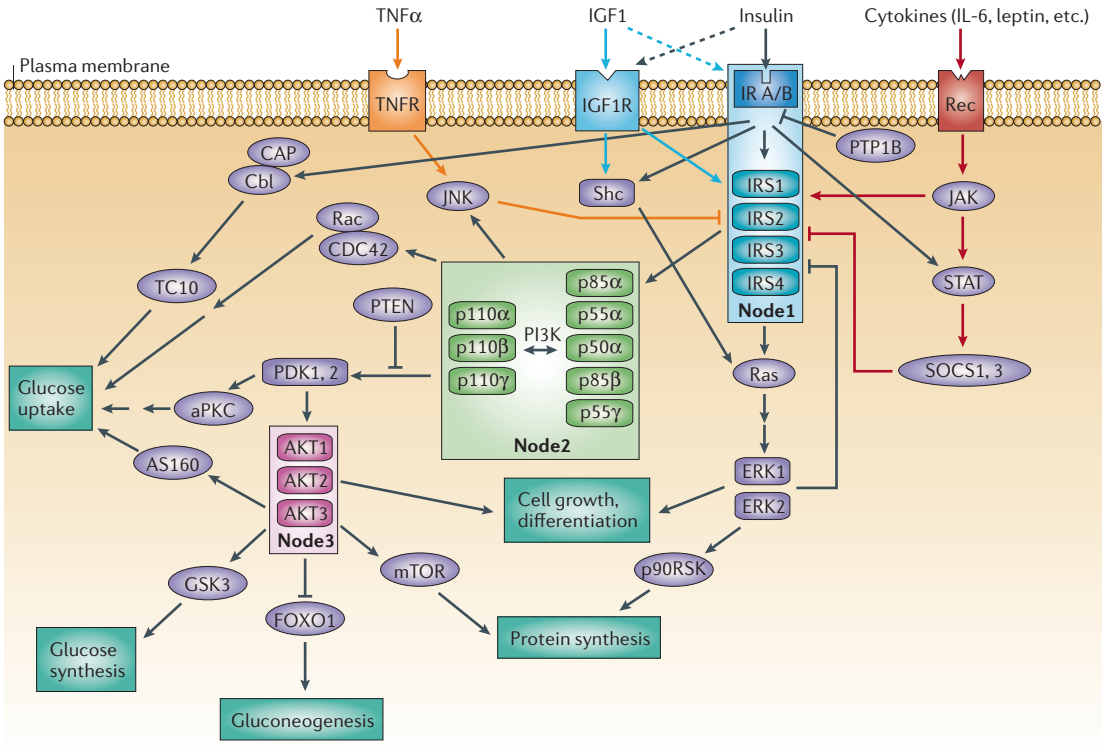

Figure 2 | **Critical nodes in the insulin-signalling network.** Critical nodes form an important part of the signalling network that functions downstream of the insulin receptor (IR) (black arrows) and the insulin growth factor-1 receptor (IGF1R) (blue arrows). Signalling pathways that are activated by cytokines such as tumour necrosis factor-α (TNFα), interleukin-6 (IL-6), and leptin interfere with insulin signalling through crosstalk (orange and red arrows). Three important nodes in the insulin pathway are the IR, the IR substrates (IRS) 1–4 (light blue box), the phosphatidylinositol 3-kinase (PI3K) with its several regulatory and catalytic subunits (light green box), and the three AKT/protein kinase B (PKB) isoforms (pink box). Downstream or intermediate effectors, as well as modulators, of these critical nodes include atypical protein kinase C (aPKC), Akt substrate of 160 kDa (AS160), Cas-Br-M (murine) ecotropic retroviral transforming sequence homologue (Cbl), Cbl-associated protein (CAP), cell-division cycle 42 (CDC42), extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2), forkhead box O1 (FOXO1), glycogen synthase kinase 3 (GSK3), Janus kinase (JAK), c-Jun-N-terminal kinase (JNK), mammalian target of rapamycin (mTOR), p90 ribosomal protein S6 kinase (p90RSK), phosphoinositide-dependent kinase 1 and 2 (PDK1 and 2), phosphatase and tensin homologue (PTEN), protein tyrosine phosphatase-1B (PTP1B), Ras, Rac, Src-homology-2-containing protein (Shc), suppressor of cytokine signalling (SOCS), signal transducer and activator of transcription (STAT), and Ras homologue gene family, member Q (ARHQ; also called TC10). Dashed arrows represent an activation process with less intensity.

Pleckstrin-homology domain
(PH domain). A protein domain of ~100 amino acids with homology to pleckstrin that mediates the binding to membrane phospholipids, such as PIP_3.

Phosphotyrosine-binding domain
(PTB domain). A domain that mediates the binding to specific phosphotyrosine residues. The affinity of a PTB domain for a phosphotyrosine depends on the residues that surround the phosphotyrosine. For instance, the PTB domain of the IRS proteins binds to an NPXpY motif.

Src-homology-2 domain
(SH2 domain). A domain of ~100 amino acids that binds to phosphotyrosine residues in proteins. Every protein's SH2 domain might have a slightly different phosphotyrosine-binding motif.

The best-studied SH2 proteins that bind to phosphorylated IRS proteins are adaptor molecules, such as the regulatory subunit of PI3K, or the adaptor molecule Grb2, which associates with son-of-sevenless (SOS) to activate the Ras–MAPK pathway (reviewed in REF. 15). The other category of proteins that bind to IRS proteins are enzymes, such as SH2-domain-containing tyrosine phosphatase-2 (SHP2)[16], and cytoplasmic tyrosine kinases, such as Fyn. There are also a few proteins that bind to IRS proteins that do not contain known SH2 domains, such as the calcium ATPases SERCA1 and 2 (REF. 17), and the SV40 large T antigen[18]. Like the IR, the ability of the IRS proteins to mediate intracellular signalling is also regulated by the action of tyrosine phosphatases. Interestingly, SHP2, which binds to IRS1 on two phosphotyrosine residues at its C terminus[16], dephosphorylates the other phosphotyrosines that mediate the binding of PI3K and Grb2.

Serine phosphorylation of IRS proteins. IRS proteins also undergo serine phosphorylation in response to insulin and other stimuli, including cytokines and free fatty acids[19]. There are over 70 potential serine-phosphorylation sites in IRS1, and in general, serine phosphorylation seems to negatively regulate IRS signalling. Serine phosphorylation of IRS1 is increased in insulin-resistant states, and might have a role in the pathogenesis of insulin resistance. Many IRS kinases, such as extracellular signal-regulated kinase (ERK)[20], S6 kinase[21], and c-Jun-N-terminal kinase (JNK)[22], are activated by insulin, which indicates that serine phosphorylation of the IRS proteins might represent a negative-feedback mechanism for the insulin-signalling pathway. Serine phosphorylation of the IRS proteins might also be a mechanism that mediates crosstalk between signalling systems. Recently, the activation of nuclear factor (NF)-κB-mediated pathways also have been shown to inhibit insulin signalling through enhanced serine phosphorylation of IRS1 (REF. 23).

Box 1 | **The structure of the insulin receptor**

The insulin receptor (IR) is a tetrameric protein that consists of two extracellular α-subunits and two intracellular β-subunits. It belongs to a subfamily of receptor tyrosine kinases (RTKs), which also includes the insulin growth factor-1 receptor (IGF1R) and an orphan receptor, known as the IR-related receptor (IRR)[120]. Each of these receptors is the product of a separate gene, in which the two subunits are derived from a single-chain precursor or proreceptor that is processed by a furin-like enzyme to give a single α–β-subunit complex. Two of the α–β dimers are linked with disulfide bonds to form the tetramer, thereby allowing for the creation of a hybrid IR–IGF1R complex, which serves as an additional receptor isoform.

Functionally, the IR behaves like a classical allosteric enzyme in which the α-subunit inhibits the tyrosine-kinase activity that is intrinsic to the β-subunit. Insulin binding to the α-subunit, or removal of the α-subunit by proteolysis or genetic deletion, leads to a derepression — for example, activation of the kinase activity in the β-subunit[121]. Following this initial activation, there is transphosphorylation of the β-subunits, which leads to a conformational change and further increase in kinase activity.

In addition, the α-subunit undergoes differential mRNA splicing into two isoforms that either lack (IR_A) or contain (IR_B) exon 11, which encodes 12 amino acids near the C terminus of the α-subunit[122]. The IR_A and IR_B isoforms have differential expression patterns, and the IR_B isoform has a higher affinity for IGFII binding[123]. Moreover, in pancreatic β-cells, IR_A and IR_B have been linked to different downstream events that are associated with transcription of insulin and cell survival[124].

The exact mechanisms by which serine phosphorylation alters IRS1 function are not clear. One possible mechanism is that the phosphorylated serine interferes with the functional domains of IRS1 in which they reside (FIG. 3). For instance, the phosphorylation of Ser307 of IRS1, which is located in the PTB domain, has been correlated with negative regulation of insulin signalling[24]. *In vitro* phosphorylation of this residue is sufficient to disrupt the interaction between the IR and IRS1 in a yeast-tri-hybrid assay[25]. Phosphorylation of Ser270, also within the PTB domain, has been shown to promote the interaction of IRS1 with 14-3-3 proteins, which might interfere with the function of the PTB domain[26]. In addition, a recent *in vivo* study has shown that decreased phosphorylation at Ser612 by MAPK correlates with increased phosphorylation of Tyr608 (REF. 27).

Although serine phosphorylation of IRS1 strongly correlates with insulin resistance, its exact role in the pathophysiology of insulin resistance is still not completely understood. Moreover, it is still unknown whether the serine phosphorylation of other IRS proteins might also have an important regulatory role.

Regulation of IRS expression. The IRS proteins might also be regulated by decreased levels of protein expression. Hyperinsulinaemia is known to decrease the expression of IRS1 and IRS2 in both cell-culture models and in the tissues of mice[28]. Two possible mechanisms could explain this effect. First, hyperinsulinaemia induces degradation of IRS1 protein and inhibits the synthesis of IRS2 at the transcriptional level[28]. Second, some studies have shown that SOCS proteins might induce ubiquitin-mediated degradation of IRS1 and IRS2 (REF. 29). Regardless of the mechanism, decreased levels of IRS proteins, coupled with decreased levels of the IR itself, certainly contribute to the insulin resistance in diabetic states in both rodents and humans[30].

Transphosphorylation
The transfer of a phosphate by a protein kinase to a residue within the same kinase molecule, or to a different kinase molecule of the same kind.

Insights from knockout studies. Although the IRS proteins are highly homologous and possess many similar tyrosine-phosphorylation motifs, studies in knockout mice and knockout cell lines indicate that the various IRS proteins serve complementary, rather than redundant, roles in insulin/IGF1 signalling (reviewed in REF. 31) (FIG. 4). *Irs1*-knockout mice have defective insulin action primarily in the muscle, and a generalized defect in body growth due to IGF1 resistance[32]. *Irs2*-knockout mice have greater defects in insulin signalling in the liver, and show altered growth in only a few tissues, such as certain neurons[33] and pancreatic β-cells[34]. Likewise at the cellular level, *Irs1*-knockout pre-adipocytes have defects in differentiation[35], whereas *Irs2*-knockout pre-adipocytes differentiate normally, but fail to respond to insulin-stimulated glucose transport[36]. These knockout studies clearly indicate that the IRS proteins constitute a critical node, such that the deletion of each isoform has a different biological consequence.

The mechanisms that account for differences between IRS isoforms, particularly IRS1 and IRS2, have recently been explored both *in vitro* and *in vivo*. When small interfering RNAs (siRNAs) are used to reduce the expression of IRS1 or IRS2 specifically in L6 myotubes, IRS1 is found to more closely regulate glucose uptake, whereas IRS2 seems to be more closely linked to MAPK activation[37]. Tissue-specific knockdown of hepatic IRS1 and IRS2 using adenoviral short hairpin RNAs *in vivo* has demonstrated that IRS1 and IRS2 have complementary roles in the maintenance of AKT/PKB activation, but exert differential roles in the regulation of gene expression[38]. Therefore, decreased hepatic IRS1 correlates with the increased expression of genes that are involved in gluconeogenesis, whereas downregulation of hepatic IRS2 results in enhanced expression of genes that are involved in lipogenesis.

Biochemical studies have revealed several molecular mechanisms by which the IRS proteins could exert their specific effects. For instance, IRS1 and IRS2 have been shown to differ in their ability to bind to various SH2 partners. IRS1 can bind to the Abl tyrosine kinase and SHP2, whereas IRS2 does not[39]. In addition, IRS1 was shown to bind to several SH2 proteins with greater affinity than IRS2, including Grb2, the Crk adaptor protein and phospholipase Cγ[39]. IRS1 and IRS2 have also been shown to have a differential ability to activate various members of the atypical protein kinase C (aPKC) family[40]. IRS3 and IRS4 probably modify the actions of IRS1 and IRS2, as they cannot activate MAPK and PI3K to the same degree as IRS1 and IRS2, and might actually antagonize some of their functions when expressed at high levels[41]. The IRS-protein isoforms also differ in their cellular compartmentalization[42] and activation kinetics[43]. In addition, IRS2 is structurally different from the other IRS proteins — it can bind to the IR through a unique kinase-regulatory-loop binding domain, which might contribute to its specific effects[44].

The PI3K node

The PI3K enzyme consists of a regulatory and a catalytic subunit, each of which occurs in several isoforms (FIG. 5).

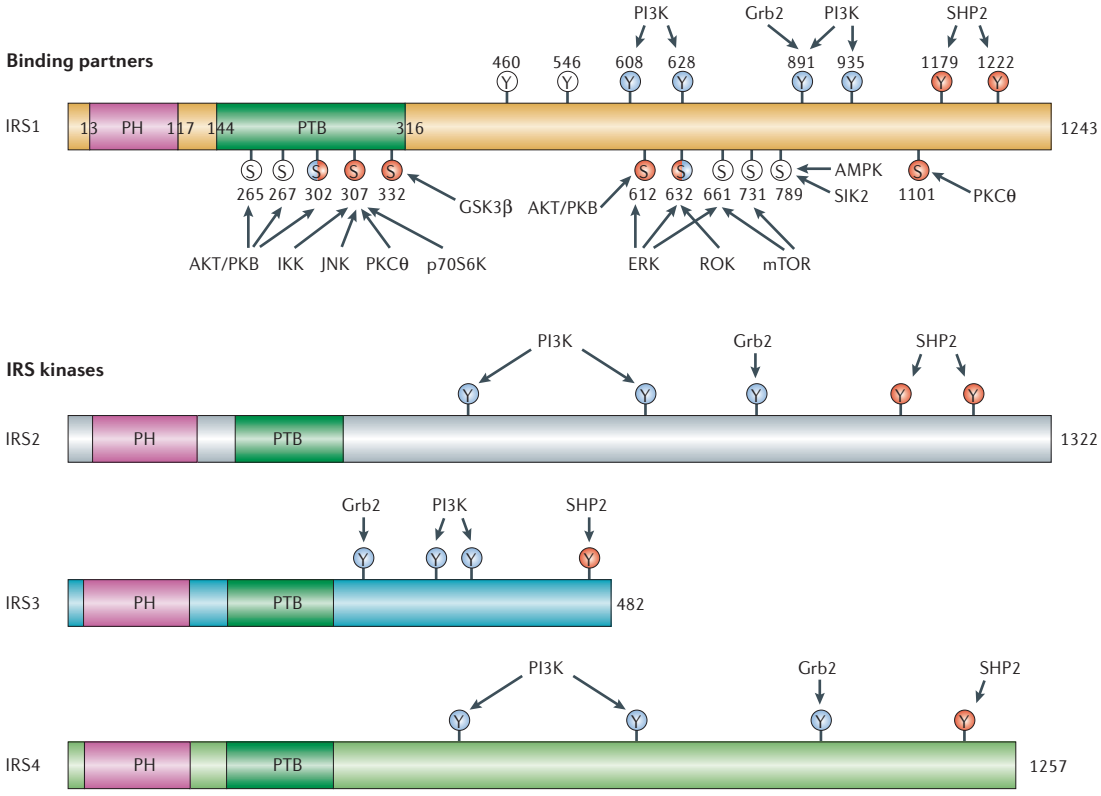

Figure 3 | **Structure and interacting partners of the insulin-receptor substrates.** The four insulin-receptor substrate (IRS) isoforms, IRS1, IRS2, IRS3 and IRS4, share a pleckstrin-homology (PH) domain (magenta), a phosphotyrosine-binding (PTB) domain (dark green) and several sites of phosphorylation on tyrosine and serine residues. The positions of the tyrosine residues (Y) that are phosphorylated by the IR and the downstream-signalling proteins that bind to these sites are shown. The positions of the serine residues (S) and the kinases responsible for their phosphorylation are also shown. Blue circles represent sites of positive regulation, whereas red circles represent sites of negative regulation. A combination of both colours shows sites in which the regulation has been reported to be either positive or negative under various conditions. White circles represent sites in which the effect of phosphorylation is currently unknown. Several proteins bind to or phosphorylate IRS, including phosphatidylinositol 3-kinase (PI3K), growth-factor-receptor-bound protein-2 (Grb2), Src-homology-2 (SH2) domain-containing tyrosine phosphatase-2 (SHP2), AKT/protein kinase B (PKB), IκB kinase (IKK), c-Jun-N-terminal kinase (JNK), protein kinase Cθ (PKCθ), p70 ribosomal protein S6 kinase (p70S6K), glycogen synthase kinase-3 β (GSK3β), extracellular signal-regulated kinase (ERK), Rho kinase (ROK), mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), salt-inducible serine/threonine kinase 2 (SIK2).

Phosphotyrosine motif
A phosphorylated tyrosine residue that is flanked by a few different amino-acid residues. Different phosphotyrosine-binding proteins have varying affinities for different phosphotyrosine motifs, which explains, in part, the specificity of phosphotyrosine-binding proteins.

AGC kinases
A large family of serine/threonine kinases that includes members as diverse as the cAMP-dependent protein kinases, and protein kinase C. The members of this AGC subgroup include AKT/PKB, serum/glucocorticoid kinases and the atypical PKCs.

The activation of the catalytic subunit depends on the interaction of the two SH2 domains in the regulatory subunit, with specific phosphotyrosine motifs in the IRS proteins of the sequence (pY)MXM and (pY)XXM[45]. Inhibitors of PI3K or transfection with dominant-negative constructs block almost all of insulin's metabolic actions, including stimulation of glucose transport, glycogen synthesis, lipid synthesis and adipocyte differentiation[46], which highlights the pivotal role of this enzyme in the metabolic actions of insulin (FIG. 4).

PI3K regulates insulin signalling by generating PIP_3. PI3K activates critical regulators of insulin signalling by catalysing the formation of the lipid second messenger PIP_3 in the cell (FIG. 5). Proteins with PH domains can bind to PIP_3 and become localized to the same region, which allows for their activation. The AGC superfamily of serine/threonine protein kinases, guanine-nucleotide-exchange proteins of the Rho family and the TEC family of tyrosine kinases are among these proteins. Perhaps the most critical of the AGC kinases for insulin action is the 3-phosphoinositide-dependent protein kinase 1 (PDK1), which is responsible for the activation of AKT/PKB and aPKCs. PDK1 phosphorylates the activation loops of AKT/PKB on Thr308 (REF. 47) and PKCζ on Thr410 (REF. 48), enhancing the activity of these kinases. AKT/PKB, however, requires a second phosphorylation on Ser473 for full activation. Because PDK1 is unable to phosphorylate Ser473, the existence of a PDK2 has been postulated. Recently, new evidence has revealed that the PDK2 for AKT/PKB might be the rapamycin-insensitive companion of mTOR (rictor)–mammalian target of rapamycin (mTOR) complex. This complex is necessary for Ser473 phosphorylation in *Drosophila melanogaster* and mammalian cells, including adipocytes[49].

The positive actions of PI3K can be negatively regulated at the level of PIP_3 by phospholipid phosphatases, such as phosphatase and tensin homologue

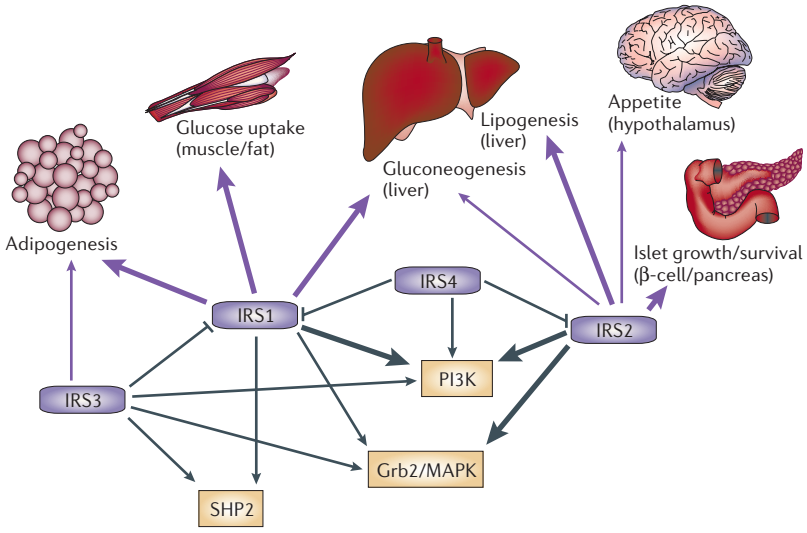

Figure 4 | **Isoform-specific functions of IRS proteins.** This figure summarizes the relative contribution of each insulin-receptor substrate (IRS) isoform (purple boxes) to the biological actions that are regulated by insulin (black arrows), as determined by knockdown and knockout studies. The main signalling pathways that are activated by each of these isoforms are also shown. These pathways involve Src-homology-2 (SH2)-domain-containing tyrosine phosphatase-2 (SHP2), growth-factor-receptor-bound protein-2/mitogen-activated protein kinase (Grb2/MAPK) and phosphatidylinositol 3-kinase (PI3K). The thickness of the arrows is proportional to the intensity of the signal that is mediated by the IRS proteins.

(PTEN) and SH2-containing inositol 5′-phosphatase-2 (SHIP2), which dephosphorylate and inactivate PIP_3. PTEN dephosphorylates phosphoinositides on the 3′-position[50], whereas SHIP2 functions on the 5′-position. *In vivo* deletions of PTEN improve insulin sensitivity[51], whereas the role of SHIP2 is controversial[52]. However, a recent knockout of SHIP2 protects mice against obesity-induced insulin resistance[53].

Several non-redundant isoforms of the PI3K subunits. PI3K exists as a heterodimer that consists of a catalytic subunit of 110 kDa and an SH2-containing regulatory subunit of ~85 kDa. The three different catalytic subunits, p110α, β and δ are derived from three different genes and show different tissue distribution, with the α and β forms being almost ubiquitous (reviewed in REF. 54), and p110δ being restricted to leukocytes. The catalytic subunit of PI3K is almost always found bound to the regulatory subunit, because free p110 is unstable and quickly degraded[55]. This binding of p110 to the regulatory subunits not only stabilizes the catalytic subunit, but also allosterically inhibits enzymatic function until the heterodimer binds to phosphotyrosines, which relieves the inhibition[55]. The p110α and p110β knockouts are early embryonic lethal, which indicates that each of these isoforms has a unique biological function that cannot be compensated for by the expression of the others. Overexpression studies have indicated that p110α might have a more important role in platelet-derived growth-factor signalling, whereas p110β is more important in mediating insulin-stimulated glucose uptake[56].

The catalytic subunit of PI3K also regulates its own function through its intrinsic serine-kinase activity that phosphorylates the regulatory subunit p85α on Ser608 (REF. 57). This phosphorylation is known to decrease the enzymatic activity of the heterodimer. In addition, PI3K can serine-phosphorylate IRS1 and decrease its signalling in cell-culture models[58].

At least eight isoforms of the regulatory subunit of PI3K have been identified, which are derived from three distinct genes[59]. *Pik3r1* encodes 65–75% of the regulatory subunits, mostly in the form of p85α, and is also responsible for producing the splice isoforms p55α and p50α. Each of the *Pik3r1* gene products can also be expressed with or without a spliced 24-nucleotide insert[59]. p85α is expressed ubiquitously, whereas p55α and p50α are expressed primarily in skeletal muscle and liver, respectively. *Pik3r2* produces p85β and accounts for roughly 20% of the regulatory subunits in the cell[60]. *Pik3r3* encodes p55PIK, which is similar in structure to p55α, but is expressed at low levels in most tissues.

In addition to its role as a positive regulator of PI3K function, the regulatory subunit is also recognized as an important negative regulator of PI3K and insulin action. This notion stems from the paradoxical observation that knockouts of the regulatory subunit can improve insulin sensitivity[60–63], and can rescue the diabetic phenotype of mice with genetic reductions of IR and IRS1 (REF. 64). This inverse correlation between p85 levels and insulin sensitivity is also applicable when the regulatory subunits are expressed at greater-than-normal levels. For instance, p85 overexpression is correlated with skeletal-muscle insulin resistance in mouse models of gestational diabetes[65], and has been linked to insulin resistance in obese humans[66].

Negative regulation of insulin signalling by PI3K. The negative regulation of insulin signalling by the PI3K regulatory subunits is thought to occur through several mechanisms (FIG. 5). One factor is the stoichiometry of the regulatory subunit to the catalytic heterodimer[64]. Under normal conditions, the concentration of regulatory subunits is in excess of the catalytic subunits and phosphorylated IRS proteins. This catalytically inactive monomeric p85 competes with the p85–p110 heterodimer for binding to the phosphotyrosines on the IRS proteins. Therefore, a reduction of the regulatory subunit can improve insulin action because it preferentially reduces the p85α monomer, and allows the heterodimer to bind to phosphorylated IRS proteins. This mechanism, however, does not fully explain the negative effects of the regulatory subunit, because the overexpression of the short isoforms p55α or p50α do not achieve similar decreases in insulin action despite similar alterations in stoichiometry[67].

At least two other mechanisms that involve only full-length p85 might also help to explain the negative effects of the regulatory subunits. The first involves the sequestration and/or compartmentalization of PI3K activity. For example, it has been shown that free, monomeric p85α, and not other regulatory subunit isoforms, can

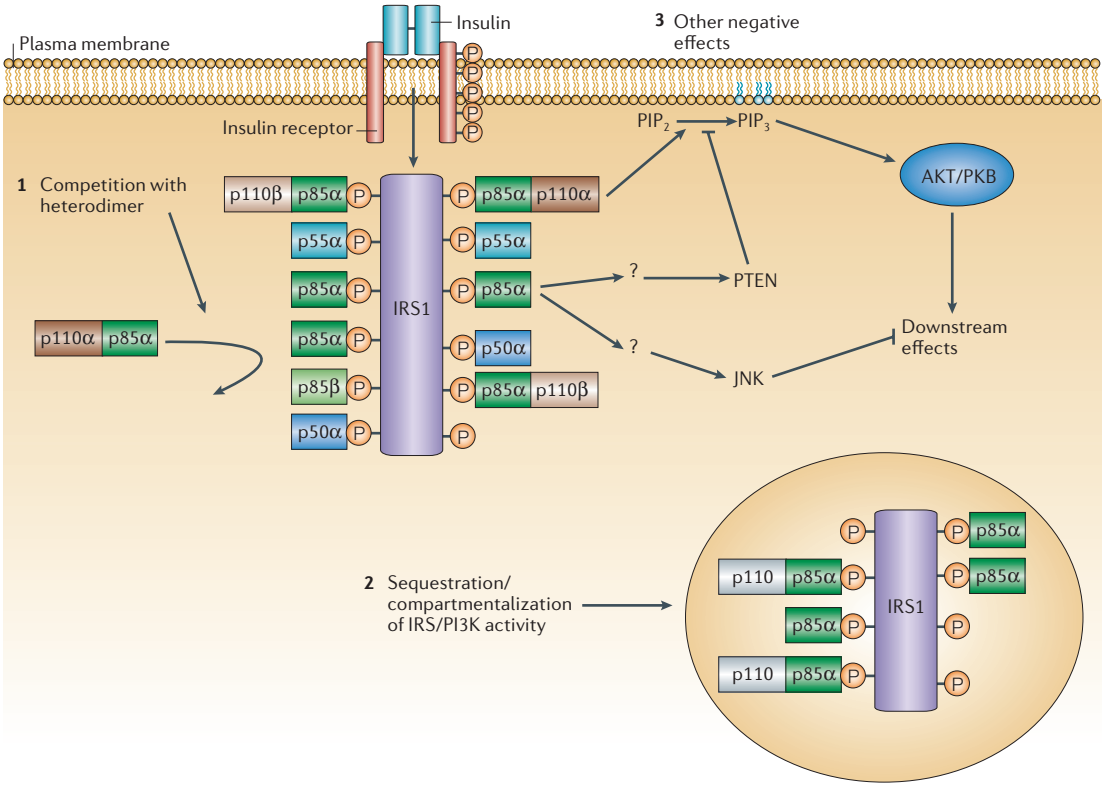

Figure 5 | **PI3K as a critical node.** The combinations of the phosphatidylinositol 3-kinase (PI3K) derive from dimerization of the different PI3K regulatory and catalytic subunits. The binding of the heterodimers to phosphotyrosines (P) on insulin-receptor substrate (IRS) proteins leads to the production of phosphatidylinositol-3,4,5-triphosphate (PIP_3) from PIP_2 at the plasma membrane. This phospholipid triggers activation of further downstream-signalling cascades that are mediated by AKT/protein kinase B (PKB). The higher abundance of the regulatory subunits compared to heterodimers allows for competition for the binding to the IRS (**1**), and diminishes efficient signalling. Other mechanisms of regulation involve the sequestration of the IRS/PI3K complex in a signalling-silent compartment (**2**) and the recruitment of inhibitory proteins that might interact with the p85 regulatory subunits. This could then lead to the degradation of PIP_3 by phospholipid phosphatases, such as the phosphatase and tensin homologue (PTEN), as well as negative regulation through c-Jun-N-terminal kinase (JNK) (**3**).

Src-homology-3 domain (SH3 domain). A protein sequence of ~50 amino acids that facilitates the binding to proline-rich regions of proteins. Binding of an SH3 domain to a proline-rich region can occur in an intramolecular or intermolecular fashion.

Breakpoint-cluster region homology domain
A ~150-residue region of p85α with homology to the breakpoint-cluster region gene and homology to Rho-GTPase activating proteins, which decrease the activity of small GTPases. Despite this homology, the breakpoint-cluster region homology domain has not been demonstrated to have GTPase activity.

sequester IRS1, and any associated PI3K enzymatic activity, into inert cellular foci that are incapable of generating PIP_3 (REF. 68).

The second mechanism involves crosstalk between the p85 subunit and the stress-kinase pathway. Recent studies have shown that the p85 subunit is required for the insulin-stimulated activation of JNK. Reconstitution studies indicate that the regulation of JNK activity is mediated through p85α, and not the shorter *Pik3r1* isoforms. JNK activity is also restored by a dominant negative p85 that cannot bind to p110, which indicates that monomeric p85 activates JNK independent of its role as a component of the PI3K holoenzyme[69]. We have recently generated a conditional knockout of *Pik3r1* in liver, and found that this relationship between p85α and JNK also occurs *in vivo*, and might have an important role in the regulation of whole-body insulin sensitivity (C.M.T., J. Luo, L. Cantley, C.R.K., unpublished observation).

The negative regulation that occurs almost exclusively by the p85 regulatory subunit indicates that the functional differences might stem from structural differences between the isoforms (FIG. 5). All the regulatory subunit isoforms share a common C terminus with two SH2 domains and an inter-SH2 domain, which has the p110 binding domain (reviewed in REF. 70). The structure of the subunits, however, diverges in the length and composition of their N termini. The N termini of p85α and p85β are ~339 amino acids long and contain an SH3 domain, two proline-rich domains and a breakpoint cluster-region homology domain. p55α and p50α have N-terminal sequences that consist of only 34 amino acids and 6 amino acids, respectively[59].

The unique N terminus of p85α has been found to bind to several interesting molecules that might regulate insulin sensitivity, including c-Cbl[71] and the small GTPases Rac1 and cell-division cycle 42 (CDC42)[72]. Although the *in vivo* effect of these interactions on insulin sensitivity has yet to be determined, the preponderance of evidence indicates that p85α probably has many ways of regulating insulin sensitivity, besides its conventional role in regulating the catalytic subunit of PI3K.

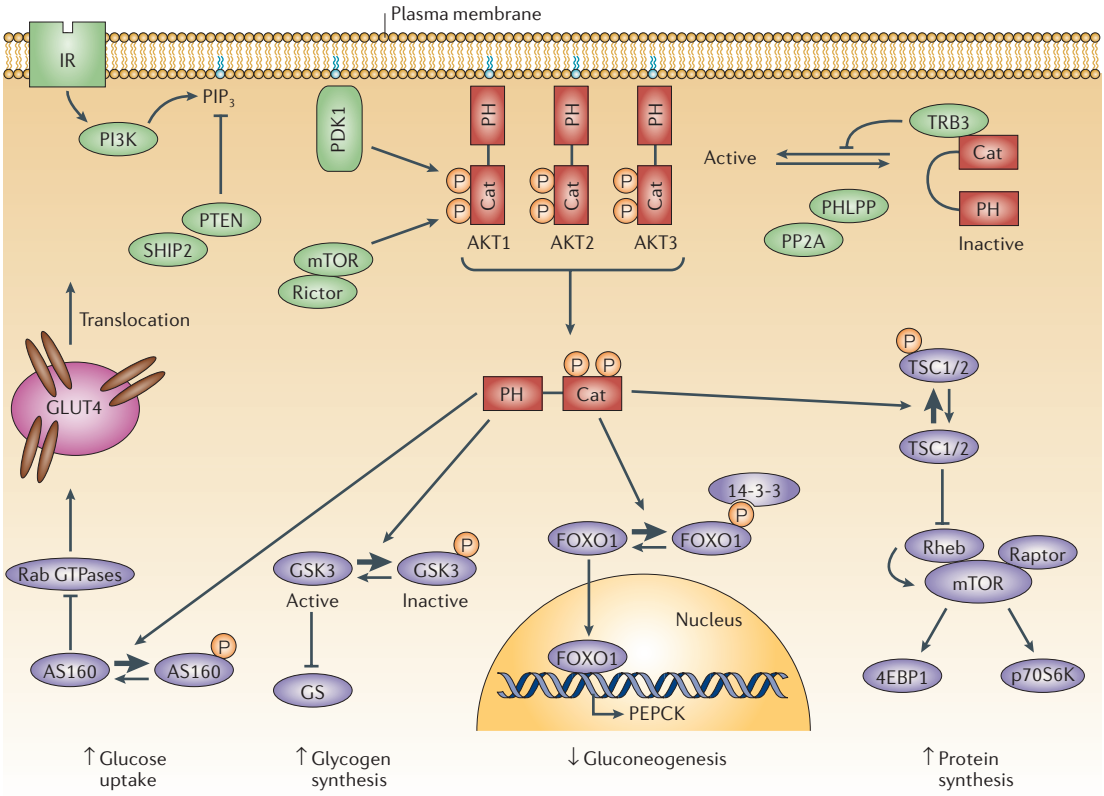

Figure 6 | **AKT/PKB as a critical node.** The three AKT/protein kinase B (PKB) isoforms (in red) share the same structural organization: a pleckstrin-homology (PH) domain, which is required for binding to phospholipids, and a catalytic domain (Cat), which becomes active on phosphorylation (P) of two residues (Tyr308 and Ser473 for AKT1, Tyr309 and Ser474 for AKT2, and either Tyr305 alone or Tyr305 and Ser472 for AKT3). The upstream regulators of AKT/PKB activity are represented in green. Positive regulators are phosphatidylinositol 3-kinase (PI3K), which produces phosphatidylinositol-3,4,5-triphosphate (PIP_3) in response to insulin. PIP_3 recruits phosphoinositide-dependent kinase-1 (PDK1) and AKT/PKB at the plasma membrane, where AKT/PKB is phosphorylated by PDK1 and PDK2 (this is thought to be a complex between the mammalian target of rapamycin (mTOR) and the rapamycin-insensitive companion of mTOR (rictor)). Negative regulators include the phosphatases phosphatase and tensin homologue (PTEN), Src-homology-2 domain-containing inositol phosphatase-2 (SHIP2), phosphatase-2A (PP2A) and the PH-domain leucine-rich-repeat protein phosphatase (PHLPP). Tribbles-3 (TRB3) is another protein that can bind to and inhibit AKT/PKB. The downstream targets of AKT/PKB are shown in purple. The GTPase activating protein AKT substrate of 160 kDa (AS160) is one of the AKT/PKB targets that is responsible for the translocation of the glucose transporter-4 (GLUT4). Glycogen synthase kinase-3 (GSK3), forkhead box O1 (FOXO1) and the tuberous sclerosis complex-1 and -2 (TSC1/2) complex are direct targets of AKT/PKB. GSK3 inhibits glycogen synthase (GS). Phosphorylated FOXO1 is sequestered in the cytoplasm on binding to 14-3-3 proteins. TSC1/2 inhibits the small GTPase Ras homologue enriched in brain (Rheb), an activator of mTOR. mTOR, associated with regulatory associated protein of mTOR (raptor), phosphorylates its substrates eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and p70 ribosomal protein S6 kinase (p70S6K). Plain arrows represent an activation process, and blocked arrows represent an inhibition process. IR, insulin receptor; PEPCK, phosphoenolpyruvate carboxykinase.

AKT/PKB as a critical node

AKT/PKB is a serine/threonine kinase that is a downstream target of PI3K signalling. AKT/PKB mediates most of the PI3K-mediated metabolic actions of insulin through the phosphorylation of several substrates, including other kinases, signalling proteins and transcription factors (FIG. 6).

AKT/PKB targets. Glycogen synthase kinase-3 (GSK3) was the first physiological target of AKT/PKB to be identified[73]. Phosphorylation of GSK3 decreases its activity towards glycogen synthase, which leads to increased glycogen synthesis[74]. GSK3 can also phosphorylate several other substrates and is involved in many processes besides the regulation of glycogen synthesis[74].

AKT/PKB seems to regulate glucose uptake by phosphorylating and inhibiting the Rab-GTPase-activating protein, AS160 (for AKT substrate of 160 kDa)[75]. This triggers the activation of Rab small GTPases that are involved in the cytoskeletal re-organization that is required for the translocation of the glucose transporter GLUT4 to the plasma membrane. AKT/PKB also phosphorylates and inhibits tuberin (also known as tuberous sclerosis complex-2, TSC2), which is in complex with hamartin (TSC1)[76]. Because this complex inhibits the growth regulator mTOR, the negative regulation of TSC1/2 by AKT/

PKB effectively activates the mTOR pathway. mTOR associates with raptor and other proteins, and regulates protein synthesis by phosphorylating the proteins p70 ribosomal protein S6 kinase and eukaryotic translation initiation factor 4E binding protein-1 (REF. 76).

AKT/PKB also regulates the expression of gluconeogenic and lipogenic enzymes by controlling the activity of the winged helix or forkhead (FOX) class of transcription factors. The FOX family contains over 100 members, and several of these are crucial for insulin action[77]. For instance, FOXO1 activates gluconeogenic genes in liver[78] and inhibits adipogenesis[79], and insulin reverses these effects through AKT/PKB, which phosphorylates FOXO1 on Ser256. This event leads to interactions with 14-3-3 proteins and sequestration in the cytoplasm[77]. The same principle applies to FOXA2, which is a crucial regulator of fasting lipid metabolism. AKT/PKB-mediated phosphorylation of Thr156 of FOXA2 prevents its nuclear localization and transcriptional activity[80].

Complex regulation of AKT/PKB. AKT/PKB activity is regulated by several inhibitory molecules, including enzymes like the protein phosphatase-2A (PP2A) and the PH-domain leucine-rich repeat protein phosphatase (PHLPP), which directly dephosphorylate and deactivate AKT/PKB[81,82]. Other regulators, such as tribbles-3 (TRB3), bind to unphosphorylated AKT/PKB and inhibit its phosphorylation and activation *in vivo*[83]. TRB3 levels are upregulated in fasting mice, and overexpression of TRB3 increases hepatic glucose output. Conversely, RNAi-mediated downregulation of TRB3 improves insulin sensitivity[84].

Divergent functions of the three AKT/PKB isoforms. AKT/PKB has three isoforms in mammals, each encoded by a different gene (AKT1–3, also known as PKB α, β and γ; FIG. 6). These isoforms share the same general structure that consists of an N-terminal PH domain and a C-terminal catalytic domain, both with high degrees of amino-acid identity. Nevertheless, recent data that were obtained using knockout mice and siRNA indicate that the isoforms are involved in the regulation of different biological processes. Deletion of AKT1 results in growth retardation and reduced lifespan[85], but no metabolic abnormalities[86]. By contrast, AKT2-deficient mice display insulin resistance and develop diabetes due, at least in part, to the inability of insulin to induce glucose utilization and decrease hepatic glucose output[87]. Consistent with these studies, a mutation in the kinase domain of AKT2 has been found to cause severe insulin resistance and diabetes in humans[88]. AKT3, on the other hand, does not seem to have a role in glucose homeostasis, but has significant effects on neural development[89].

The specificity of action of different AKT/PKB isoforms results from differences in their tissue distribution, subcellular localization and downstream-signalling targets. The relative ratios of expression of the three isoforms vary considerably among tissues. AKT3 is predominantly expressed in the nervous system and testis, whereas AKT1 and AKT2 are widely distributed, with AKT2 particularly enriched in insulin-sensitive tissues, such as liver and fat[90]. Studies done in adipocytes derived from *Akt2* knockout animals have shown that insulin-stimulated glucose uptake is severely reduced and cannot be compensated for by overexpression of AKT1 (REF. 91). An siRNA-based approach in 3T3-L1 adipocytes has also highlighted the primary role of AKT2 on insulin responsiveness in adipocytes[92]. Interestingly, AKT2 has been found to be co-localized with GLUT4-containing vesicles, a property not shared by AKT1 and AKT3 (REF. 93). Furthermore, Synip, a protein that regulates the docking and fusion of GLUT4-containing vesicles, is phosphorylated by AKT2, but not by AKT1 or AKT3 (REF. 94). Because the main structural differences between AKT isoforms occur at the PH domains[95], it is tempting to speculate that other AKT targets will also show isoform specificity based on the differential binding of their PH domains to various phospholipids or other binding partners.

Other possible signalling nodes

The IR/IRS, PI3K and AKT/PKB signalling nodes are 'critical nodes' because they fulfil the criteria for being a critical node (as discussed above), including a significant amount of *in vitro* and *in vivo* evidence. Below we present some other molecules that have been implicated in insulin action. Although they have some of the characteristics of critical nodes, more experiments are required to define their true role in the signalling network, and to determine whether they represent true critical nodes.

aPKCs and glucose transport. Along with AKT/PKB activation, an important downstream effector of PI3K in mediating the metabolic effects of insulin is the activation of aPKC. In contrast to conventional or novel PKCs, aPKCs are not sensitive to the second messengers calcium or diacylglycerol (DAG). As a member of the AGC superfamily, the mechanisms of the aPKCs are similar to AKT/PKB, requiring PIP_3 and phosphorylation by PDK1 (REF. 96). aPKCs have been shown to have a role in insulin-stimulated glucose uptake and GLUT4 translocation in adipocytes and muscle[97]. Decreased activation of aPKCs has been reported in the muscle of type-2 diabetic humans and rodents, whereas AKT/PKB activation might be unchanged[98]. The aPKCs have also been involved in the regulation of lipid synthesis in the liver by increasing the level of sterol-regulatory-element binding protein-1c expression[98].

The aPKC isoenzymes λ and ζ are encoded by two different genes, and their levels of expression vary. PKCλ is the main aPKC in the skeletal muscle and adipose tissue of the mouse, whereas PKCζ is more prominent in rats, monkeys and humans[97]. The high level of positive and negative regulation of these kinases, and the role that these proteins have in insulin action, indicate that they might form a critical node in insulin signalling. However, *in vitro* reconstitution experiments have indicated that both isoforms can function interchangeably to support insulin-stimulated glucose transport[97]. Tissue-specific knockouts of the various aPKC isoforms might shed light on the relative roles of these genes.

Diacylglycerol
(DAG). DAG is a lipid second messenger that is produced by the cleavage of PIP_2 by phospholipase C. DAG is involved in the activation of conventional and novel protein kinase Cs (PKCs).

GLUT4 translocation
Relocalization and fusion of an intracellular vesicle that contains the glucose transporter GLUT4 to the plasma membrane. It allows the uptake of extracellular glucose that is stimulated by insulin in muscle and adipose tissues.

The Ras–MAPK pathway. The Ras–MAPK pathway is activated by insulin following the binding of Grb2 and the guanyl nucleotide-exchange factor SOS to cognate phosphotyrosines on IRS proteins Shc and Gab1. This binding triggers the activation of the small GTPase Ras and the subsequent activation of Raf, which triggers a kinase cascade that results in the phosphorylation and activation of the dual-specificity kinases MEK1 (MAPK and ERK kinase 1) and MEK2, which in turn phosphorylate MAPK/ERK1 and ERK2 on threonine and tyrosine residues. The activated ERKs phosphorylate various targets, including p90 ribosomal protein S6 kinase (p90RSK) and the transcription factor ELK1, therefore promoting gene expression[99]. The Ras–MAPK pathway is mainly involved in mediating cell growth, survival and cellular differentiation. Although it has been postulated that ERK1 and ERK2 have similar functions, and ERK2 is able to compensate for ERK1 loss in *Erk1* knockout mice[100], ERK1 has been shown to be specifically required for adipogenesis *in vitro* and *in vivo*[101]. ERK1 and ERK2 have also been involved in a negative-feedback loop of insulin action by phosphorylating IRS1 on serine residues[20]. Therefore, although ERK1 and ERK2 might have some of the features of critical nodes, the preliminary *in vivo* evidence indicates that these kinases might only be necessary for the growth and differentiation aspects of insulin actions, and not the other metabolic effects.

As with many components of signalling networks, both Ras and the Raf kinases come in several isoforms. The three isoforms of Ras, H-Ras, N-Ras and K-Ras are ubiquitously expressed. The different lipid anchors on the three Ras isoforms have the potential to target them into different environments and different signalling complexes[102]. Also, the p85/p110–PI3K complex binds to Ras, linking two pathways that are normally considered to be distinct[103]. The Raf proteins also consist of three isoforms: A-Raf, B-Raf and C-Raf, which are differentially regulated[104]. Although all are able to activate MAPK, there are differences in the duration or the intensity of activation that might influence the cellular response[104]. However, so far, no differential impact on insulin action has been observed.

Rho family of GTPases. The small GTPases of the Rho family, Rac and CDC42, have also been implicated in insulin action. The two Rac isoforms (1 and 2) are encoded by two separate genes[105], whereas the two CDC42 isoforms are produced by alternative splicing[106]. Both Rac and CDC42 are activated by the exchange of GDP to GTP, which triggers a conformational change that induces the interaction of the GTPases with downstream effectors, many of which are involved in the rearrangement of the actin cytoskeleton. This property is thought to link Rac to GLUT4 translocation in skeletal muscle[107], whereas CDC42 might have this role in adipocytes[108].

Stress-activated p38 MAPK and JNK. Three JNK-coding genes (*JNK1–3*) have been described in mammals, each with several splice variants. JNK1 and JNK2 are expressed ubiquitously, whereas JNK3 expression is restricted to neuronal tissues[109]. Insulin has been shown to activate JNK1 and JNK2 in various cellular systems[22]. As mentioned previously, JNK activation might negatively regulate insulin signalling through serine phosphorylation of IRS1. Interestingly, JNK activity has been found to be increased in insulin-resistant states, including obesity and inflammation, and deletion of JNK1, but not JNK2, improves insulin sensitivity in obese mice[24], which indicates a role in the pathophysiology of disease.

p38 MAPK is also an intracellular target of insulin. The four splice variants of the p38 family (p38α, β, γ and δ) share 60% identity and have different tissue distribution, with p38α and β being ubiquitously expressed[110]. Several studies indicate that p38 MAPK activation is required for full GLUT4 translocation[111], however, p38 MAPK activation has also been reported to have contradictory roles in GLUT4 expression in various tissues[112]. Whether insulin differentially affects the activation of various isoforms of p38 MAPK, or if these variants specifically modulate distinct cellular processes, is unknown.

The CAP–Cbl pathway. The c-Cbl proto-oncogene is tyrosine-phosphorylated by the IR, and forms a complex with other molecules such as Cbl-associated protein (CAP) that results in the activation of the small GTPase TC10 (REF. 13). It has been suggested that the CAP–Cbl pathway collaborates with the PI3K pathway in the stimulation of GLUT4 translocation[113], although this is still being debated. The Cbl family contains the three members c-Cbl, Cbl-b and Cbl-3 that have been shown to have a role in the ubiquitylation of receptor tyrosine kinases[114]. Interestingly, c-Cbl-deficient mice have improved insulin sensitivity that is associated with reduced adiposity and higher energy expenditure[115]. Clearly, further investigation is required to determine the role of Cbl proteins in insulin action.

Conclusions

As our understanding of the genome and proteome grows, so does the complexity of cell-signalling networks, which underscores the importance of establishing a framework to define the critical nodes in the system, and their roles in the network. The insulin-signalling network illustrates well the concept of critical nodes, as the molecular relationships between many of its components have been validated using genetic and biochemical approaches *in vitro* and *in vivo*. These studies demonstrate the essential features of a critical node. Critical nodes consist of several molecular isoforms that are involved in divergent signalling, they are highly regulated (both positively and negatively), they are essential for the biological actions of the ligand, and in many cases, they are points of crosstalk with other signalling systems. These nodes allow for the incredible diversification and fine-tuning of insulin's signal in normal physiology and disease states.

The main challenge to validating the critical nodes of other ligand–receptor systems is the *in vivo*

elucidation of the exact roles of each component. Genetic techniques, such as knockout by homologous recombination, provide one approach to uncovering the unique biological functions of different signalling isoforms. The use of RNAi might also enable the rapid assessment of gene-isoform function and regulation, both *in vitro*[116,117] and *in vivo*[38,118], as could other emergent knockout techniques[119]. In this manner, critical-node analysis is not only helpful in understanding insulin signalling, but will also be a broadly useful concept in dissecting the biological effects of all receptor–ligand-mediated processes.

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Competing interests statement
The authors declare no competing financial interests.

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