Review

MOLECULAR METABOLISM

Insulin action at a molecular level – 100 years of progress

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ABSTRACT

The discovery of insulin 100 years ago and its application to the treatment of human disease in the years since have marked a major turning point in the history of medicine. The availability of purified insulin allowed for the establishment of its physiological role in the regulation of blood glucose and ketones, the determination of its amino acid sequence, and the solving of its structure. Over the last 50 years, the function of insulin has been applied into the discovery of the insulin receptor and its signaling cascade to reveal the role of impaired insulin signaling—or resistance—in the progression of type 2 diabetes. It has also become clear that insulin signaling can impact not only classical insulin-sensitive tissues, but all tissues of the body, and that in many of these tissues the insulin signaling cascade regulates unexpected physiological functions. Despite these remarkable advances, much remains to be learned about both insulin signaling and how to use this molecular knowledge to advance the treatment of type 2 diabetes and other insulin-resistant states.

Keywords Insulin; Insulin receptor; Insulin signal transduction; Insulin resistance

1. INTRODUCTION

The discovery of insulin 100 years ago and its application to the treatment of human disease in the years since have marked a major turning point in the history of medicine [1]. Amazingly, insulin was not initially recognized as a peptide hormone, and virtually nothing was known about its mechanism of action. This all dramatically changed 50 years ago with the identification of the insulin receptor, initially through binding studies [2] and then, 10 years later, by the recognition that the receptor was a tyrosine kinase [3]. We now understand the importance of insulin and insulin-like growth factor signaling systems as integrators of metabolism, growth, and lifespan in species from *C. elegans* to *Homo sapiens*. In this brief review, we summarize the nature of this insulin signaling system and how knowing this system has led to better understanding of insulin-resistant states and diabetes. It is also important to realize that despite huge advances, much remains to be learned about both insulin signaling and how to use this knowledge to advance the treatment of type 2 diabetes and other insulin-resistant states.

2. INSULIN RECEPTOR TYROSINE KINASE

At the cellular level, insulin initiates action by binding to its membrane receptor. The insulin receptor (InsR) is encoded by a 150-kb gene composed of 22 exons on human chromosome 19p13.3-p13.2. During synthesis, a single-chain InsR proreceptor is formed, glycosylated, linked into a dimer by disulfide bonds, and cleaved to generate the α- and β-subunits that create the $\alpha_2\beta_2$ tetramer (Figure 1) [4,5]. The extracellular domain is composed of the entire α-subunit and the NH_2-terminal portion of the β-subunit. The α-subunit contains two leucine-rich (L1 and L2) repeats flanking a cysteine-rich region (CR). These are followed by three fibronectin-III (Fn_{iii}) motifs, which are interrupted by a 120 amino acid insert domain (ID) that contains the furin cleavage site allowing separation of the α- and β-subunits (Figure 1).

One of the remarkable insights of the past few years has been visualization of the receptor in its three-dimensional structure. X-ray crystallography and cryo-EM studies reveal that the unoccupied extracellular domain folds into an inverted V, with the apex formed by the L2 and $Fn_{iii}1$ of each α-subunit (Figure 1) [6]. Mutagenesis and affinity-labeling studies predict two insulin binding sites in the α-subunit: a high-affinity site-1, created by the L1 domain from one α-subunit and the C-terminus of the other α-subunit, and site-2, located near the $Fn_{iii}1 \rightarrow Fn_{iii}2$ interface (Figure 1) [6–10]. Exon-11 of the InsR gene is alternatively spliced to produce two isoforms (IR-A, which omits exon-11, and IR-B, which includes exon-11), adding 12 amino acids at the C-terminus of the α-subunit [11]. This constitutes part of the insulin binding domain, thus increasing the affinity for insulin [12,13]. These two forms of InsR and the homologous IGF1R can form heterodimers, allowing a total of five receptor "subtypes," which interact with insulin, IGF-1, and IGF-2 with differing affinities.

Even before its cloning, studies using patient-derived InsR autoantibodies revealed that the InsR β-subunit was a tyrosine kinase [3]. This was a remarkable observation, since at that time, only the epidermal

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Abbreviations

AC	adenylate cyclase
ACADL	acyl-CoA dehydrogenase long chain
ACACA	acetyl-CoA carboxylase
ACh	acetylcholine
AKT	V-Akt Murine thymoma viral oncogene homolog
AMPK	adenosine monophosphate (AMP) activated kinase
aPKC	atypical protein kinase C
ARHGEF	Rho Guanine Nucleotide Exchange Factor
AS160	Akt substrate of 160 kDa (also called TBC1D4)
ATGL	adipose triglyceride lipase
CaN	Calcineurin
CAP	Cbl-associated protein
CBL	Cas-Br-M (murine) ectopic retroviral transforming sequence
ChREBP	carbohydrate response element binding protein
CREB	cyclic AMP (cAMP) response element binding protein
CRL7	Cullin—RING-type E3 ligase 7
CRTC2	CREB regulated transcription coactivator 2
CUL5	Cullin 5
CUL7	Cullin 7
DAG	diacylglycerol
ELOB	Elongin B
ELOC	Elongin C
ERK1/2	Mitogen-activated protein kinase $^{1}/_{2}$
FA	fatty acid
FBW8	F-Box and WD repeat domain containing 8
FOXA2	hepatocyte nuclear factor 3-beta
FOXK	Forkhead box protein K1
FOXK2	Forkhead box protein K2
FOXO1	Forkhead box protein O1
G3P	glycerol-3-phosphate
G6P	Glucose-6-Phosphate
G6Pase	glucose-6-phosphatase
GLP1	glucagon-like peptide 1
GLP1R	glucagon like peptide 1 receptor
GLUT2	glucose transporter 2
GLUT4	glucose transporter 4
Gq/G11/Ga	G-proteins
GRB2	Growth factor receptor bound protein 2
GSK3β	glycogen synthase kinase 3 beta
HDAC	Histone deacetylase
HNF1B	hepatocyte nuclear factor 1-beta
HSL	hormone sensitive lipase
IKKα	Inhibitor of nuclear factor kappa B kinase subunit alpha
IKKβ	Inhibitor of nuclear factor kappa B kinase subunit beta
IL6	Interleukin 6
INFγ	Interferon Gamma
INPPL1	Inositol Polyphosphate Phosphatase Like 1 (SHIP2)
JAK	Janus kinase
JNK	C-Jun N-Terminal Kinase 1
KDM	Lysine demethylase
KAT	Lysine acetyltransferase
M3R	muscarinic 3 receptor
MAPK	mitogen-activated protein kinases
MEF2C	Myocyte enhancer factor 2C
MEK1	Mitogen-activated protein kinase kinase 1
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
NAD^{+}	oxidized nicotinamide adenine dinucleotide
NEMO	Inhibitor of nuclear factor kappa B kinase regulatory subunit
NFAT	Nuclear factor of activated T-cells
NGN3	neurogenin 3
PC1/3	Prohormone convertase 1/3
PDE3B	phosphodiesterase 3B
PP2A	PP1, pTEN, C1-TEN and SHIP2. I
PDHA1	Pyruvate Dehydrogenase E1 Subunit Alpha 1
PDK1	phosphoinositide-dependent kinase-1
PDX1	pancreatic and duodenal homeobox 1
PEPCK	phosphoenolpyruvate carboxykinase
PI(3,4,5)P3	phosphatidylinositol (3,4,5)-trisphosphate
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLCβ	phospholipase C
PPP1CA (PP1)	Protein Phosphatase 1 Catalytic Subunit Alpha
PPP2CA (PP2A)	Protein Phosphatase 2 Catalytic Subunit Alpha
PPP1R12A	Myosin phosphatase-targeting subunit 1 (MYPT1)
PTEN	phosphatase and tensin homolog
PTPN1 (PTP1B)	Protein Tyrosine Phosphatase Non-Receptor Type 1
PTPN2 (TCPTP)	Protein Tyrosine Phosphatase Non-Receptor Type 2
RAF	Raf proto-oncogene, serine/threonine kinase
RBX1	Ring-Box 1
RHEB	Ras homolog, mTORC1 binding
RIP1	Receptor interacting serine/threonine kinase 1
RNU1-1	RNA U1 Small Nuclear 1 (U1)
RNU2-1	RNA U2 Small Nuclear 1 (U2)
SETD	SET domain containing histone lysine methyltransferase
SHC	SH3-containing protein
SOS1	son of sevenless Ras/Rac guanine nucleotide exchange factor 1
SR	Serine and arginine rich splicing factor
SREBF1	sterol responsive element binding factor 1
TAK1	Transforming growth factor-beta-activated kinase 1
TBC1D1	TBC1 (Tre-2/USP6, BUB2, Cdc16) Domain Family, Member 1
TC10	Ras Homolog Gene Family, Member Q
TG	triglyceride
TNFα	Tumor necrosis factor alpha
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAF	TNF receptor associated factor 1
TSC1	Tuberous Sclerosis 1 Protein (Hamartin)
TSC2	Tuberous Sclerosis 2 Protein (Tuberin)

growth factor receptor was known to have this enzymatic activity. Two groups sequenced the InsR cDNA four years later to confirm this discovery [14,15]. Structurally, the intracellular portion of the β-subunit is composed of three regions, each containing clusters of tyrosine autophosphorylation sites: Y_{965} and Y_{972} (numbered as in IR-B) in the juxtamembrane domain (JMD), Y_{1158}, Y_{1162} and Y_{1163} in the activation loop of the kinase domain, and Y_{1328} and Y_{1334} in the carboxy-terminus [16,17] (Figure 1). Insulin binding to sites-1 and -2 converts the ECD into a T-conformation that promotes a convergence of the intracellular domains, thus facilitating full activation of the receptor kinase toward exogenous substrates by tyrosine transphosphorylation [[18] and article by Lawrence in this issue].

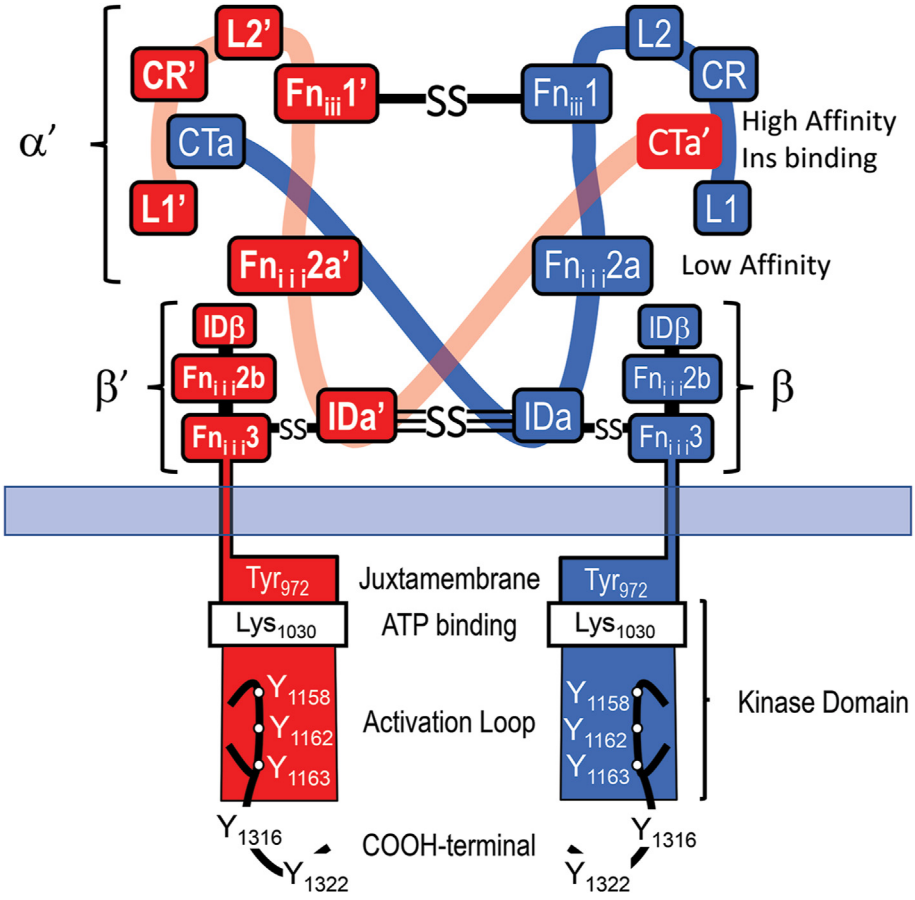

Figure 1: Schematic diagram of the mature insulin receptor, composed of two extracellular α-subunits (red and blue) and two covalently linked transmembrane β-subunits. Contiguous modules of the α-subunits are labeled with the relative location of disulfide bonds (S—S) between the α- and β-subunits. The high-affinity insulin binding site is created from the L1-CR and CTa' or L1′-CR′ and CTa domains of the disulfide-linked α- and α′-subunits. The β-subunit is formed upon furin-mediated cleavage of the ID region into IDα and IDβ. The COOH terminus of the $Fn_{iii}2$ and $Fn_{iii}3$ domains forms after the furin cleavage site is separated from the intracellular juxtamembrane region by the hydrophobic transmembrane domain. The tyrosine kinase catalytic domain, including the canonical ATP binding site (Lys_{1030}) and the activation loop with three tyrosine phosphorylation sites, follows immediately after the juxtamembrane region. The β-subunit ends with two tyrosine phosphorylation sites in the COOH terminus.

3. THE PROXIMAL INSULIN SIGNALING CASCADE

Evidence for an insulin receptor substrate came from anti-phosphotyrosine antibody immunoprecipitates of insulin-stimulated hepatoma cells [19]. Purification and cloning of this protein revealed IRS1, the first member of the insulin receptor substrate family [20]. Two other homologous IRS proteins are present in humans (IRS2 and IRS4), and a fourth (IRS3) in rodents [21]. IRS1 and IRS2 are broadly expressed, whereas IRS3 and IRS4 are tissue-restricted [22]. All IRS proteins have tandem PH (pleckstrin homology) and PTB (phosphotyrosine binding) domains, important for membrane and receptor association, followed by a long unstructured tail containing 14 tyrosine phosphorylation sites (Figure 2). IRS proteins also contain >50 serine/threonine phosphorylation sites (shown as red diamonds in Figure 2), which modulate stability and tyrosine phosphorylation for feedback and heterologous regulation [23]. Upon insulin stimulation, the IRS proteins are recruited to a phosphorylated $NPEpY_{972}$ motif in the juxtamembrane region of the InsR [24], which facilitates phosphorylation of the tyrosine residues in the IRS tail. These in turn bind to the SH2 domains in various downstream signaling proteins, the most metabolically important of which is the p85 regulatory subunit of the class 1A PI3K (phosphatidylinositol 3-kinase) (Figure 3) [25]. Tyrosine phosphorylation of other sites in IRS-1 (or the alternative substrate SHC) recruit the Grb2•SOS complex, which activates the RAS→MAP kinase cascade, a second major branch of insulin signaling (Figure 3). The recruitment of IRS to the activated InsR/IGF1R is highly regulated and adds an essential level of signaling specificity [24,26,27].

4. THE PI3K-AKT SIGNALING CASCADE

The PI3K cascade begins when insulin stimulates the tyrosyl phosphorylation of two YMPM-motifs in IRS proteins, which bind and activate the PI3K (Figure 3) [28]. PI3K is composed of a catalytic and a regulatory subunit, each occurring in multiple isoforms encoded by multiple genes. The catalytic subunit—including p110α, p110β, p110δ, and p110γ—is stabilized and inhibited by its association with one regulatory subunit—including p85α or an alternatively spliced isoforms (p55α, p50α), p85β, or p55γ. All the regulatory subunits contain two SH2 domains that bind the phosphorylated YMPM-motifs in IRS1 or IRS2. This binding disinhibits the catalytic subunit's production of PIP3 (phosphatidylinositol 3,4,5-trisphosphate) [29—31]. Chemical or genetic inhibition of PIP3 production blocks almost all metabolic responses stimulated by insulin—including glucose uptake, glycogen and lipid synthesis, and

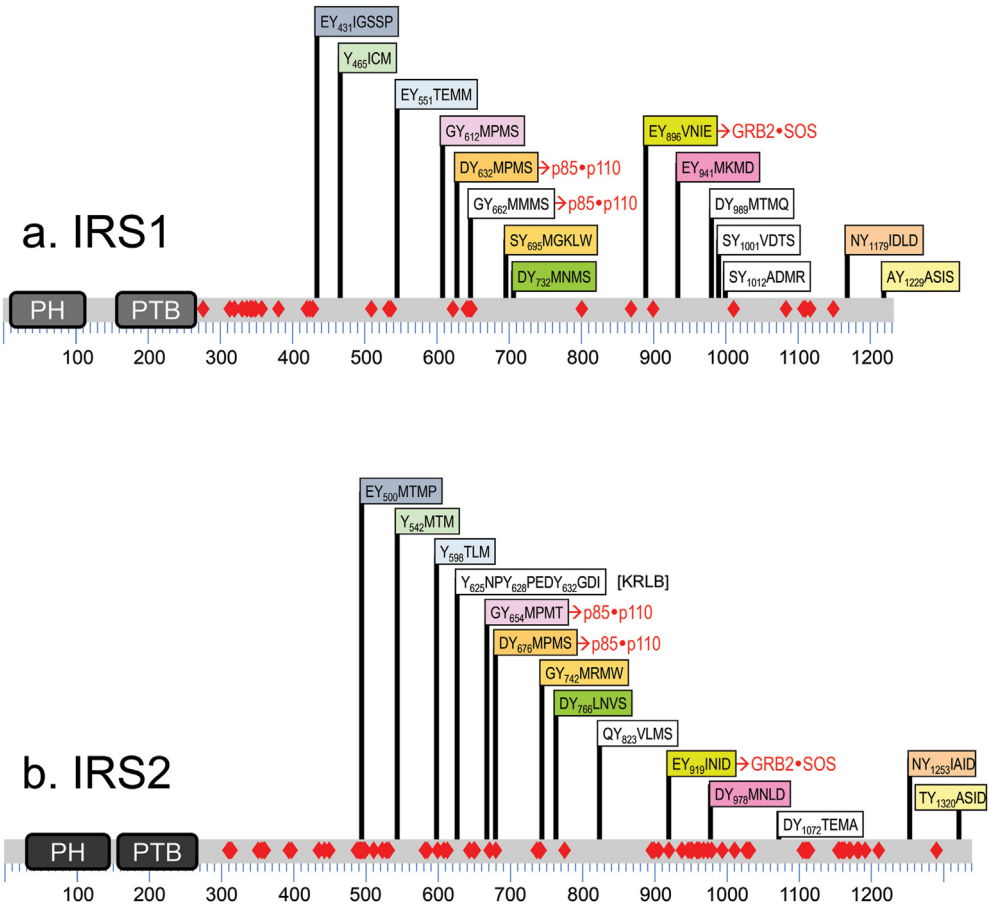

Figure 2: Comparison of (a) IRS1 and (b) IRS2. Alignments of IRS1 and IRS2 tyrosine phosphorylation sites relative to the amino-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains. Conserved tyrosine phosphorylation motifs-including their number in the human protein and the surrounding amino acid sequences-are color-coded to highlight alignments between the isoforms: white boxes indicate unique sites in IRS1 or IRS2, including the KRLB (kinase regulatory loop binding) domain in IRS2 located around Y_{624} in IRS2. The relative position of Ser/Thr phosphorylation sites in IRS1 or IRS2 revealed by MS/MS are indicated with red diamonds.

adipocyte differentiation—confirming that the PI3K is a critical node in insulin's action [32,33].

PIP3, in turn, activates PDK1 and SIN1 (also called MAPKAP1). The former phosphorylates AKT at Thr_{308}, while the latter complexes with mTORC2 to phosphorylate AKT at Ser_{473} [34]. This activates AKT, which phosphorylates consensus RXRXX (pS/pT)Ψ motifs in more than 100 substrates (Figure 3) [35]. AKT1 mainly regulates growth, development, and survival, whereas AKT2 regulates metabolism via GLUT4 translocation and glucose and lipid metabolism [36]. Humans with a dominant negative mutation in AKT2 display features of T2DM [37].

Insulin also activates mTOR (mechanistic target of rapamycin). This is a Ser/Thr kinase that forms two functionally-distinct protein complexes, mTORC1 and mTORC2, both controlled by insulin/IGF1 and other growth factors [38]. In the absence of stimulation, mTORC1 is inhibited through complexing with the TSC1•TSC2 (hamartin-tuberin) complex until AKT phosphorylates and inhibits the GTPase activity of TSC2, which activates RHEB (Figure 3). FKBP8 (FK506 binding protein 8) also inhibits mTORC1 until RHEB•GTP promotes its dissociation from mTORC1 [39]. Regulation by TSC1•TSC2 activation of RHEB is also modulated by AKT-mediated phosphorylation of AKT1S1 (PRAS40), which promotes its dissociation from RAPTOR and thus activates mTORC1, potentiating the role of insulin in growth and proliferation (Figure 3) [40]. mTORC2, on the other hand, in addition to taking part in activating AKT, plays a role in mRNA processing by phosphorylation of IMP1 (Figure 3) [41].

Another important target of AKT is the Forkhead box O family of transcription factors (FOXO1, FOXO3a, FOXO4, and FOXO6). FOXO nuclear localization is regulated by multiple posttranslational modifications, but especially AKT-mediated phosphorylation, which leads to nuclear exclusion of FOXOs following insulin stimulation, turning off their gene targets [42,43]. This leads to decreased glucose production in the liver, decreased autophagy and protein degradation in the muscle, increased adipose differentiation [44—47], and decreased hepatic IGFBP1 expression to increase circulating IGF1 bioavailability and somatic growth [48].

5. MUTATIONS IN THE INSULIN SIGNALING SYSTEM

Because of the critical nature of insulin signaling, mutations in these key proteins are rare. Regardless, the investigation of syndromes of insulin resistance have been extremely informative. Firstly, some patients have insulin resistance due to autoantibodies to the receptor, which provided the first tool for identifying receptor autophosphorylation even before its cloning [3]. Secondly, other patients with severe insulin resistance revealed the first naturally occurring mutations in the insulin receptor. These exhibit several clinical subtypes. Those with

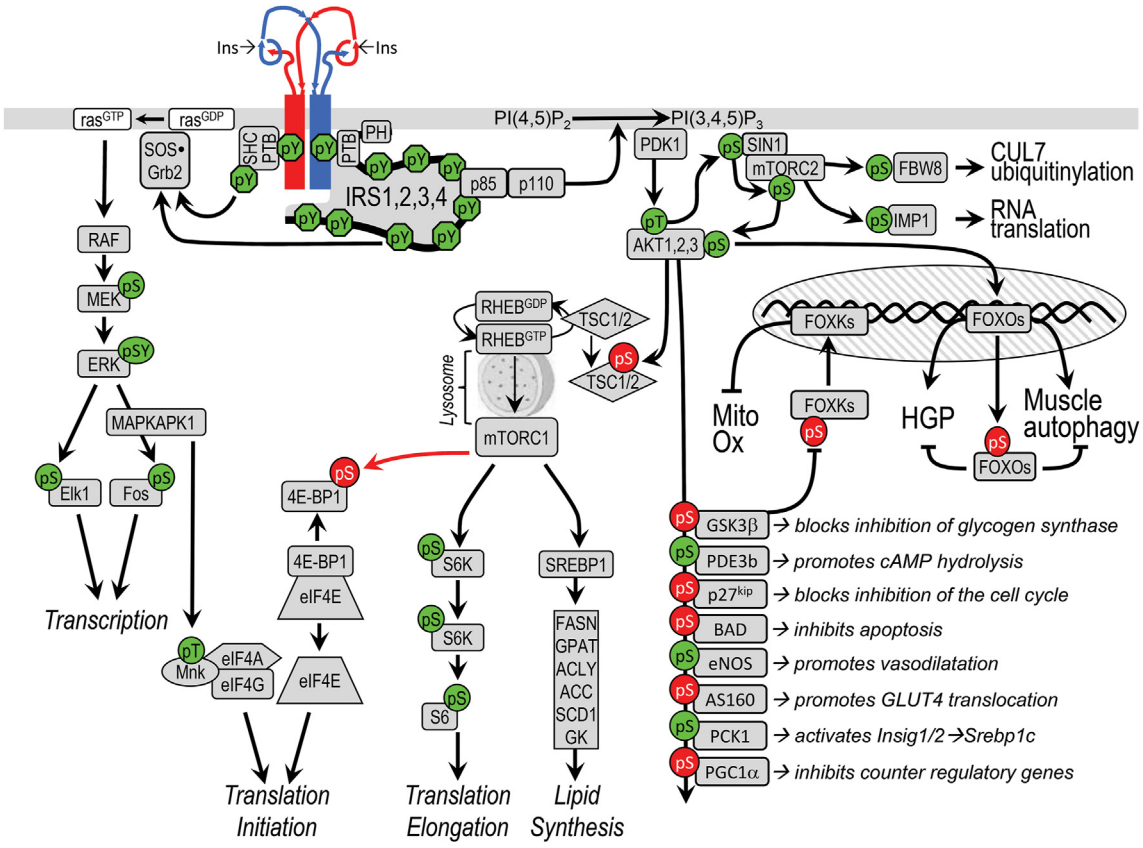

Figure 3: A canonical insulin/IGF signaling cascade. The InsR subunits are illustrated at the top in red and blue. InsR signals begin with tyrosine phosphorylation of the IRS or SHC. The IRS protein binds and activates the PI3K, which generates $PI3,4P_2$ and $PI3,4,5P_3$ that recruit PDK1, SIN1, and AKT to the plasma membrane. AKT is activated upon phosphorylation at T308 by PDK1 and at S473 by the SIN1•mTORC2 complex. mTORC1 is activated by $Rheb^{GTP}$, which accumulates upon inhibition of the GAP activity of the TSC1•TSC2 complex following AKT-mediated phosphorylation of TSC2. mTORC1-mediates phosphorylation of S6K and SREBP1, which promote protein and lipid synthesis, respectively. AKT phosphorylates many cellular proteins, inactivating PGC1α, $p21^{kip}$, GSK3β, BAD, and AS160 and activating PDE3b, PCK1, and eNOS. AKT-mediated phosphorylation of FOXO1 and FOXK causes their sequestration in the cytoplasm, which inhibits their influence upon transcriptional activity. GRB2•SOS can bind to IRS or SHC. The Grb2/SOS complex promotes GDP/GTP exchange on $p21^{ras}$, which activates the ras→raf→MEK→ERK1/2 cascade. Activated ERK stimulates transcriptional activity by direct phosphorylation of ELK1 (ETS domain-containing protein) and by indirect phosphorylation of cFOS through MAPKAPK1 (MAPK-activated protein kinase-1). MAPKAPK1 also phosphorylates other proteins, including S6 (ribosomal protein S6), NFκB, PP1, and MYT1. Insulin stimulates protein synthesis by altering the intrinsic activity or binding properties of key translation initiation and elongation factors (eIFs and eEFs, respectively) as well as critical ribosomal proteins. mTORC1-mediated phosphorylation of 4E-BP1 and S6K plays an important role in stimulating translation initiation and elongation [270]. Stimulatory phosphorylation sites are highlighted in green, and inhibitory sites are highlighted in red.

mutations in the kinase domain usually present as type A syndrome of insulin resistance and acanthosis nigricans, whereas those with mutations in the extracellular domain can have more severe phenotypes, exemplified by Donohue and Rabson-Mendenhall syndromes [49,50]. While InsR mutations can cause severe insulin resistance, leading to high insulin requirements (>10,000 U/day), some individuals remain near normoglycemic due to massive elevations of endogenous insulin secretion [49]. Loss-of-function mutations in AKT2 are extremely rare, but can also result in severe forms of insulin resistance [51–56]. A natural human variant in IRS1 with a Gly972Arg substitution that reduces insulin-stimulated PI3K signaling is relatively common [57]; however, this polymorphism plays only a minor role in T2DM risk [51–56].

6. DISSECTING THE INSULIN SIGNALING SYSTEM IN MICE BY TISSUE-SPECIFIC GENE INACTIVATION

6.1. Skeletal muscle

Using genetic approaches in mice, we and others have dissected the InsR and IGF1R signaling pathways in vivo in virtually every tissue in the body. Despite the essential role of muscle as a site for glucose uptake, as evidenced by marked hyperglycemia in muscle GLUT4 knockout mice [58,59], deletion of InsR in skeletal muscle in MIRKO mice results in only mild obesity and elevated circulating FFA and triglycerides, but without elevated glucose and insulin [53,60]. Indeed, even combined deletions of InsR and IGF1R in MIGIRKO mice results in a major reduction in muscle mass, while glucose and insulin tolerance remain normal (Figure 4A). The latter is due at least in part to increased basal glucose uptake, indicating alternative pathways for activation of glucose transport [61]. Likewise, mice with muscle-specific Irs1/Irs2 double knockout (MDKO) also fail to develop hyperglycemia, despite progressive and severe loss of skeletal and cardiac muscle due to unrestrained autophagy [62]. Again, isolated skeletal muscles from MDKO mice show elevated basal glucose uptake, elevated AMP/ATP ratio, and increased AMPK (AMP-activated protein kinase) activity (Figure 4A) [58,59,62,63]. The marked muscle atrophy in the absence of InsR and IGF1R is due to unrestrained activity of FOXOs promoting autophagy (Figure 4A). Thus, deletion of FOXO1, FOXO3, and FOXO4 can prevent muscle loss in MIGIRKO mice [64] and also prevents the muscle atrophy observed in insulin deficient diabetes [46].

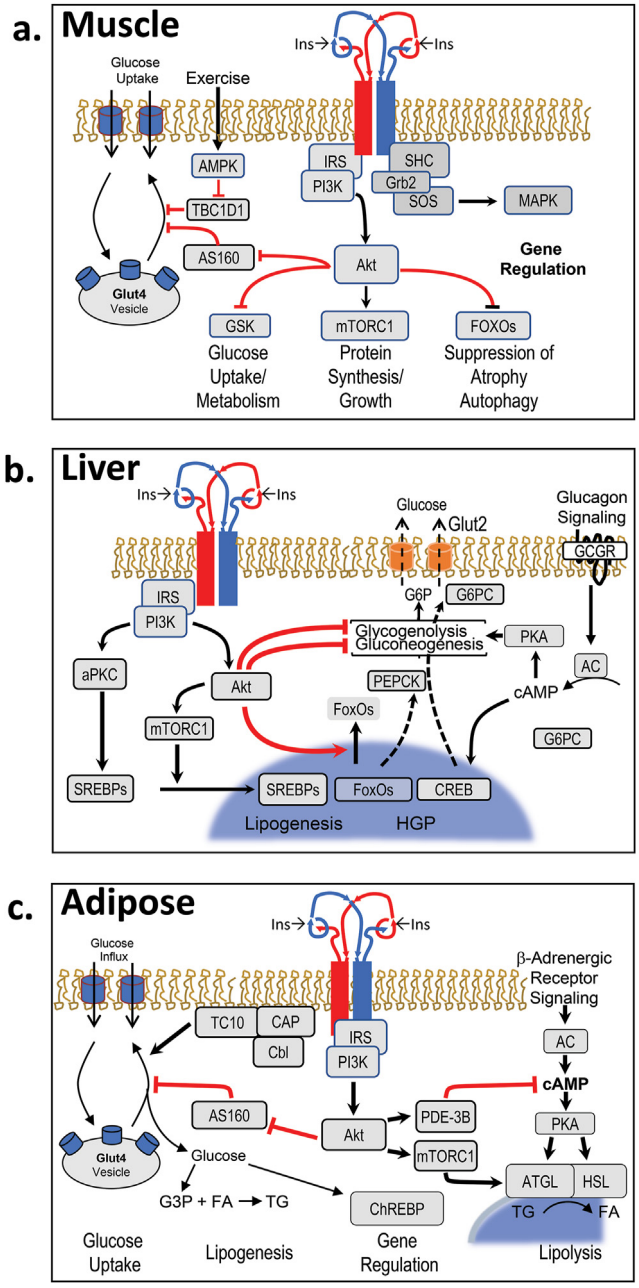

Figure 4: Tissue-specific insulin signaling. The insulin receptor is autophosphorylated on multiple tyrosine residues, allowing the docking and activation of multiple signaling molecules, most notably insulin receptor substrate (IRS) proteins. This in turn activates phosphatidylinositol-3-kinase (PI3K) and Akt to mediate the increases in glucose uptake and metabolism as well as changes in protein and lipid metabolism. While the general pathway is similar in all tissues, the final biological effects are specialized to the roles of insulin in muscle (a), liver (b), and adipose tissue (c).

6.2. Liver

Liver-specific InsR knockout (LIRKO) mice, on the other hand, display moderately elevated fasting and postprandial glucose levels and severe hyperinsulinemia [65]. The latter is due to a combination of increased insulin secretion and reduced hepatic insulin degradation [66]. LIRKO mice also display reduced levels of circulating free fatty acids (FFA) and triglycerides [53,65], and on an atherogenic diet develop dyslipidemia that can progress to atherosclerosis [67]. Insulin receptor deletion dysregulates hundreds of hepatic genes, including reduced GCK (glucokinase) and elevated PCK1 (phosphoenolpyruvate carboxykinase 1), G6PC (glucose-6-phosphatase, catalytic subunit), and PK1 (pyruvate kinase) (Figure 4B) [53,65]. The chronic hyperinsulinemia in LIRKO mice also leads to insulin resistance in other tissues. Thus, somewhat paradoxically, streptozotocin treatment to reduce insulin secretion improves peripheral insulin sensitivity [56,68–70]. Genetic inactivation of Irs1 and Irs2 or Akt1 and Akt2 in the liver in mice [56,71–73] resembles the LIRKO mouse with unsuppressed HGP, hyperinsulinemia, glucose intolerance, and diabetes, consistent with the genes' roles as critical nodes in insulin action [36,66,71–73]. As with muscle, hepatic inactivation of FOXO1 in either LIRKO or liver IRS1/2 or Akt1/2 double-knockout (DKO) mice reverses dysregulated hepatic gene expression and restores metabolic health, despite lack of upstream insulin signaling (Figure 4B) [36,56,74–76]. At least part of this improvement is due to reversing the increased levels of the FOXO1-dependent hepatokine, follistatin (Fst), which promotes WAT lipolysis and thus propagates systemic metabolic disease during hepatic insulin resistance [56].

6.3. Adipose tissue

Genetic inactivation of insulin/IGF1 signaling in adipose tissue produces different phenotypes depending on the specific promoter used to drive adipocyte Cre expression. The initial fat insulin receptor knockout mice (FIRKO) created using the aP2-Cre transgene displayed partial lipodystrophy and increased longevity [77,78], whereas FIRKO mice created using the more potent adiponectin-Cre displayed more severe lipodystrophy and NAFLD that progressed to NASH and liver dysplasia [79]. Perhaps the most interesting of these models are mice with an inducible fat-specific knockout of IR or IR/IGF1R created using the tamoxifen-regulated adiponectin-Cre [80]. Following induction of recombination, these mice rapidly develop severe lipodystrophy and systemic insulin resistance with β-cell hyperplasia; however, as new adipocytes develop from preadipocytes that have not undergone gene inactivation, this syndrome totally reverses. Finally, in recent unpublished work, Homan et al. have produced mice with a combined knockout of the IR and IGF1R as well as all three FOXO proteins expressed in fat (Foxo1, 3, and 4). As in liver and muscle, deletion of FOXOs rescues much of the phenotype created by IR/IGF1R KO, but in this case the extent of rescue depends on the adipose depot, with complete rescue of brown adipose tissue mass, partial rescue of subcutaneous adipose mass, and no rescue of perigonadal adipose mass indicating differential roles of InsR, IGF1R, and FOXO proteins in adipocyte subtypes (Figure 4C).

7. INSULIN ACTION IN NON-CLASSICAL TARGET TISSUES AS REVEALED BY GENE KNOCKOUT

7.1. Cardiovascular system

Genetic tools for tissue-specific knockout have provided unique insights into the role of insulin action in tissues not recognized as classical insulin targets. Genetic deletion of insulin and IGF-1 receptors in the heart reveals the essential role of InsR/IGF1R in cardiac development and function [81]; however, absence of InsR alone only leads to changes in potassium channel expression and ventricular repolarization [82]. Deletion of myocardial InsR also decreases VEGF expression, impairing reactive angiogenesis following ischemia reperfusion damage or myocardial infarction [83]. Similarly, muscle deletion of Irs and Irs2 causes severe left ventricular failure in mice between 3 and 4 weeks of age. At the same time, retention of a single allele of Irs1 or Irs2 can prevent sudden death, suggesting that either

can mediate enough IR/IGF1R signaling to prevent excess autophagy and fatal cardiac muscle loss [62]; however, Irs1 and Irs2 play different roles. Thus in conditions of pressure overload modeled in mice, cardiac deletion of Irs1 attenuates Akt1 activation, preventing LV remodeling and heart failure. By contrast, deletion of cardiac Irs2 leads to severe LV dysfunction that can be prevented by haploinsufficiency of Akt1 [83,84]. Therefore, InsR, Irs1, and Akt1 are critical signaling nodes required for normal LV remodeling in both mice and humans [84].

Cardiovascular disease and myocardial infarction are major causes of morbidity associated with insulin resistance and T2DM [85]. The progression of atherogenesis can involve dysregulated crosstalk between immune cells, endothelial cells (ECs), and vascular smooth muscle cells (VSMCs) [86]. Deletion of InsR or IGF1R in VSMC shows that the InsR (but not IGF1R) mediates VSMC proliferation and intimal hyperplasia following intimal injury [87]. In contrast, in ApoE-deficient mice bred with mice lacking endothelial InsR, atherosclerotic lesion size increases more than 2-fold [88]. Likewise, ApoE KO mice bred with whole-body IRS2 heterozygous KO mice have increased aortic intima thickness [89]. Endothelial cell-specific IRS2 KO also impairs eNOS phosphorylation, muscle insulin delivery, and glucose uptake [54,90], indicating a special role for IRS2 signaling in cardiovascular complications of diabetes.

7.2. Pancreatic β-cells

The capacity of pancreatic β-cells to maintain glucose homeostasis during physiologic stress is important for metabolic homeostasis. Since β-cells were thought to always be exposed to high insulin levels, β-cells were initially assumed to be non—insulin responsive, or if they did respond, this was considered part of a negative feedback loop to suppress insulin secretion [91]. However, knockout of InsR in β-cells revealed important roles of insulin signaling in both β-cell growth and glucose sensing [92], which appears to be regulated through IRS2 as mice with IRS2 KO develop β-cell failure (Figure 5) [93]. These results should still be interpreted cautiously, as these experiments were conducted with Rip-Cre mice (B6.Cg-Tg (Ins2-cre)25Mgn/J; JAX Stock No: 003573), which can express Cre at low levels in the hypothalamus [94]. Regardless, β-cell IRS2 expression is known to be regulated through the actions of FOXO1/3, NFAT, and the CREB•CRTC2 [91]. Glucose-stimulated Ca^{2+} release activates calcineurin, which dephosphorylates NFAT to facilitate its entry into the nucleus, where it induces expression of IRS2 and other genes [95]. Glucose, GLP-1 (glucagon-like peptide-1), and other GPCR agonists also promote IRS2 transcription via increased cAMP [96,97]. IRS2 expression through these mechanisms maintains PDX1 action, which is essential for β-cell growth, function, and survival (Figure 5) [98]. Compounds that augment IRS2 expression may be useful for treatment of β-cell failure during insulin resistance and the progression of T2DM [99].

7.3. Brain

Insulin resistance, obesity, and T2DM are associated with age-related cognitive decline and ADRD (Alzheimer's Disease and Related Dementia) [100—102]. How insulin resistance promotes cognitive decline, however, is still only partially understood. In the brain, insulin receptor substrates IRS1, IRS2, and IRS4 link IR-A, IGF1R, and their hybrids to downstream pathways. The neurotrophin receptors TrkA and TrkB, which regulate synaptic strength and plasticity, also engage IRSs in neurons [103,104]. Insulin action in the central nervous system and its effects on mood, behavior, and cognition are apparent in mice with brain knockout of InsR [105] or localized knockout of both insulin and IGF-1 receptors in the hippocampus and amygdala [106]. Alterations in insulin signaling proteins have also been observed in brain samples from humans with Alzheimer's Disease [101,107,108] and mouse models of AD [109], suggesting that enhancing central insulin action could be therapeutic in AD [100]. Mice with brain KO of InsR show increased tau phosphorylation [110]. Brains of AD patients show increased Ser/Thr phosphorylation of IRS1 [107,109,111]. On the other

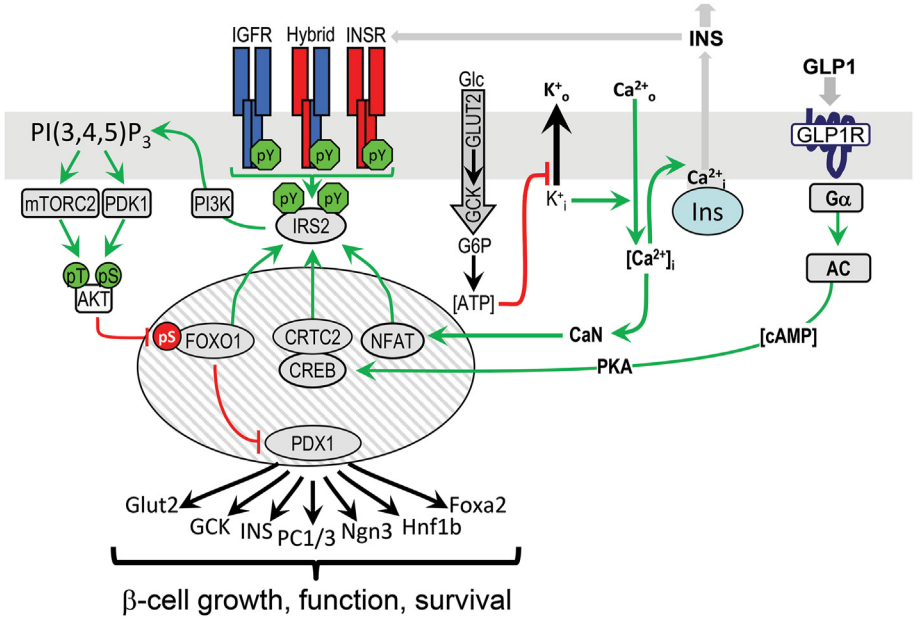

Figure 5: The integrative role of IR/IGF1R → IRS2 signaling in pancreatic β-cell function. The diagram shows the relation between the IRS2-branch of the insulin signaling pathway and upstream and downstream mechanisms regulating β-cell growth and function. Since IR and IGF1R are constitutively active in β-cells, activation of GLP1 → cAMP → PKA → CREB, glucose → Ca^{2+} → CRTC2, and calcineurin → NFAT induce IRS2 expression to regulate PI3K → AKT cascade, which places β-cell growth, function, and survival under the control of glucose and incretins.

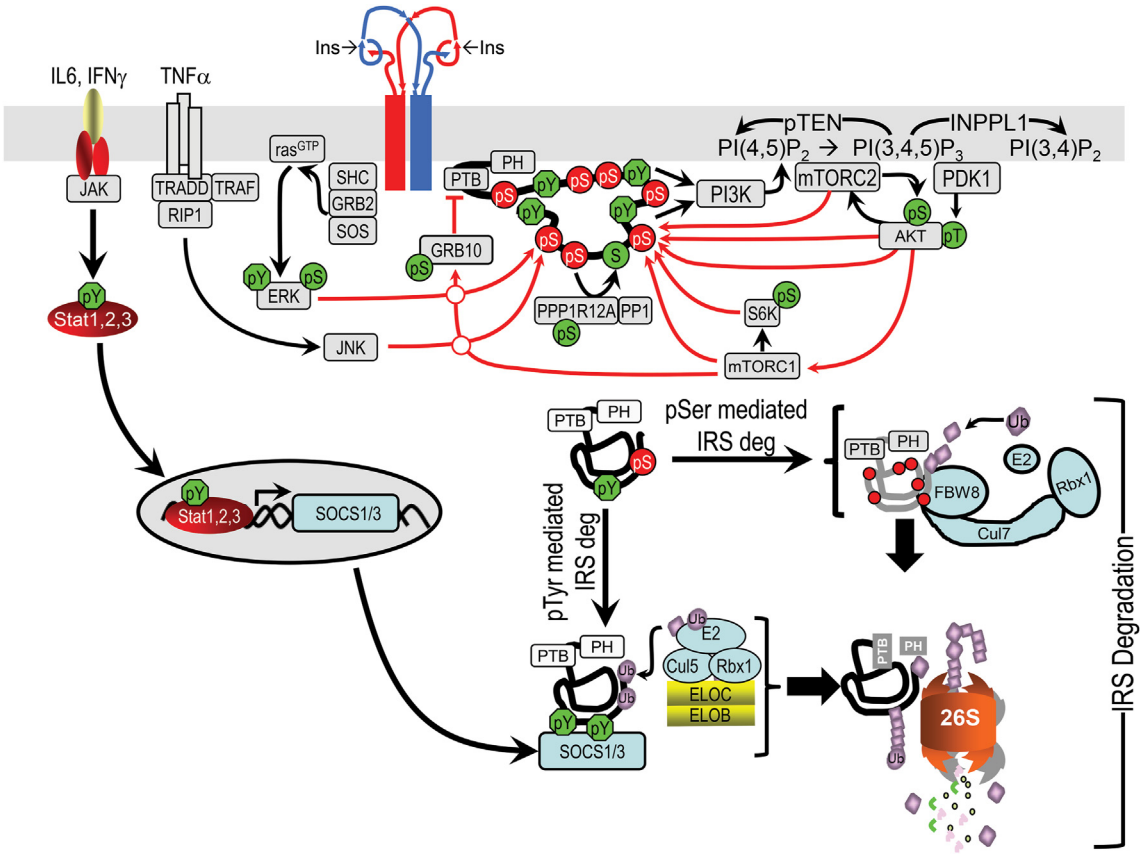

Figure 6: Schematic diagram of feedback and heterologous regulation of the insulin signaling cascade. Various kinases in the insulin signaling cascade mediate feedback of Ser/Thr phosphorylation of IRS1/2—including AKT, mTOR, S6K, ERK, and AKT [137]. Other kinases activated by heterologous signals, including IL6, INFγ, and TNFα are also illustrated. Serine phosphorylation of IRS1 can recruit CRL7, which can promote ubiquitination and degradation of IRS1 through the 26S proteasome. Many proinflammatory cytokines cause insulin resistance through SOCS1 or SOCS3, targeting phosphotyrosine-containing IRS1 or IRS2 for ubiquitination by a BC-containing ubiquitin ligase (E3) and degradation [271,272].

hand, Irs2 deficiency in the brain appears to increase lifespan and improve memory formation in mice [112—115], despite producing obesity, peripheral insulin resistance, and hyperinsulinemia [105,112,116—119]. Similarly, decreasing Irs2 improves motor performance and extends lifespan in a mouse model of Huntington's disease (R6/2), and this is associated with reduced neuronal oxidative stress and enhanced autophagy and mitochondrial function [120]. Lower Irs2 also increases nuclear FOXO1 in R6/2-mice [120], which contribute to prevention of age-related axonal degeneration [121].

8. REGULATION OF PROXIMAL INSULIN SIGNALS

Genetic experiments in mice have established that changes in a broad array of insulin signaling components, nutrient sensors, and their downstream effectors can have profound effects upon insulin sensitivity, β-cell function, and nutrient homeostasis. Indeed, a 50 % reduction in the concentration of IR and IRS1 will cause diabetes in mice [122].

8.1. Transcriptional regulation of IRS1 and IRS2

Physiologically, IRS1 expression is regulated by transcriptional repressors (including the transcription factor AP2β) or the p160 family of nuclear receptor coactivators p/CIP (p300/CBP/cointegrator-associated protein) and SRC1 (steroid receptor coactivator-1) [123,124]. Interestingly, GWAS reveals AP2β as a potential root of obesity and T2DM [125]. In contrast, p/CIP and SRC1 serve as transcriptional coactivators, and increased IRS1 expression following inactivation of p/CIP and SRC1 in mice results in increased glucose uptake and enhanced insulin sensitivity in WAT and skeletal muscle. Finally, muscle-specific knockout mice of the TAZ transcription factor display decreased IRS1 expression and insulin sensitivity [126]. Statins reduce TAZ levels, which may contribute to the insulin resistance observed in some patients on statins.

Expression of IRS2 is regulated by multiple factors, many of which respond to nutrients and energy, such as CREB (cAMP response element binding protein) and its coactivator CRTC2 [127,128]. Regulation of IRS2 by FOXO creates a direct feedback loop to augment insulin signaling during fasting. Fasting and exercise also induce the CREB•CRTC2 transcriptional complex through glucagon signaling; this increases expression of gluconeogenic genes as well as IRS2 [129]. Increases in active hepatic SREBP1c in situations of nutrient excess or chronic insulin stimulation decrease IRS2 expression, creating insulin resistance while promoting lipogenesis [130,131]. Thus, IRS2 expression is regulated through multiple metabolic sensors that modulate insulin sensitivity through feedback and heterologous mechanisms to maintain metabolic homeostasis.

8.2. Regulation of IRS degradation

IRS1 and IRS2 can also be regulated by degradation. Both proteins undergo poly-ubiquitinylation during inflammation, chronic nutrient excess, or hyperinsulinemia through various mechanisms, including SOCS1/3 and ubiquitin-mediated degradation (Figure 6) [132,133]. IRS

Ser/Thr phosphorylation (see below) also coordinates ubiquitin-mediated degradation [134,135]. Caloric excess induces CBL-Proto-Oncogene B expression, which drives insulin resistance through the polyubiquitinylation and degradation of IRS1 [136].

8.3. Regulation of IRS signaling by Ser/Thr phosphorylation

Perhaps more important than changes in protein levels, regulation of IRS1 and IRS2 occurs through a complex mechanism involving phosphorylation of more than 50 serine/threonine (Ser/Thr) phosphorylation sites, located mainly in the long unstructured tail of the molecule (See Figures 2 and 6). Understanding how phospho-Ser/Thr regulates insulin signaling, however, has been challenging because a multitude of sites and mechanisms can be involved [23]. Proinflammatory cytokines, excess free fatty acids, ceramides, amino acids and glucose, and endoplasmic reticulum stress have all been implicated in increased IRS1/2 Ser/Thr phosphorylation and reduced insulin-stimulated tyrosine phosphorylation [23]. Most IRS1/2 Ser/Thr phosphorylation is stimulated by the PI3K → Akt → mTOR cascade during insulin stimulation [23,137], suggesting that IRS1/2 phospho-Ser/Thr is likely a feedback mechanism that develops during chronic insulin stimulation and can be co-opted by metabolic stress to inhibit insulin signaling (Figure 6) [23,137]. This may also be a link between hyperinsulinemia and insulin resistance.

In mice, phosphorylation of Ser^{307} (equivalent to human IRS-1 Ser^{312}) is often used as a barometer of insulin resistance [23]. Insulin can promote IRS1-Ser^{307} phosphorylation through a pathway involving PI3K → AKT → mTORC1 → S6K1 (Figure 6) [137]. This phosphorylation is also stimulated by free fatty acids via activation of JNK1 (c-Jun N-terminal kinase) or mTORC1 [23]. Surprisingly, however, knock-in of alanine to replace Ser^{307} and eliminate the phosphorylation site increases insulin resistance, rather than decreasing it [138]. Likewise, alanine substitution at Ser^{302}, a prime target of mTORC1 → S6k mediated phosphorylation, does not prevent insulin resistance [139], indicating that other factors must be involved [132—136].

8.4. Modulation of insulin signaling by protein and lipid phosphatases

Phosphatases modulate insulin signaling by dephosphorylating key proteins or lipids in the signaling cascade. Numerous phosphatases are involved, including PTPN1 (PTP1B), PTPN2 (TCPTP), PP1, PP2A, pTEN, C1-TEN, and SHIP2. Inactivation of PTP1B, a phosphotyrosine phosphatase that can dephosphorylate InsR, increases insulin sensitivity in mice [140]. Inactivation of PTP1B maintains β-cell mass in mice lacking IRS2, preventing an early onset of diabetes [141]. Inhibition of specific hypothalamic neurons or PTP1B or TCPTP in the brain promotes insulin and leptin signaling and prevents diet-induced obesity, T2DM, and fatty liver disease [142]. Thus far, targeting PTP1B and TCPTP for treatment has been problematic due to challenges in drug development and the potential for cancer risk brought about by increasing the activity of other tyrosine kinases; however, intranasal targeting of PTP1B and TCPTP can increase leptin and insulin sensitivity and promotes weight loss by repressing feeding and increasing energy expenditure [143].

PTEN is a lipid phosphatase and a potent negative regulator of insulin action, which attenuates insulin signaling by dephosphorylating PIP3 at the 3-position, thus reducing the activation of PDK1, AKT, and other downstream molecules (Figure 6). PTEN heterozygous knockout can increase peripheral insulin sensitivity in IRS2 KO mice and normalize glucose tolerance [144]. SHIP2 (Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase) attenuates insulin signaling by dephosphorylating the 5′-position of PIP3 (Figure 6). Several genetic studies link SHIP2 to metabolic disorders, and metformin's ability to increase insulin sensitivity is at least in part through inhibition of SHIP2 [145]. Phosphoserine-directed phosphatases, including PP1 and PP2A, have complex effects depending on substrate targeting. PP1 complexed with MYPT1 that can be activated by GPCR-mediated phosphorylation removes phosphates from Ser/Thr sites in IRS1, promoting tyrosine phosphorylation to enhance insulin/IGF signaling by heterologous agonists [146].

8.5. miRNA-mediated posttranscriptional regulation

miRNAs (microRNAs) are short non-coding RNA molecules that negatively modulate gene expression through their specific binding within the 3′UTR sequence of mRNA, inhibiting translation or destabilizing the target mRNA. Most of the components of insulin signaling can be regulated in a tissue-specific way by miRNAs [147]. LET7 miRNA interferes with many proximal insulin signaling components, including IGF1R, INSR, IRS2, PIK3IP1, AKT2, TSC1, and RICTOR [148]. Other miRNAs exhibit specificity against proximal signaling molecules. miR-424—5p, miR-15b, miR-195, and miR-96 increase in mouse livers during the high-fat diet, which associates with less InsR expression [149—152]. IRS1 and IRS2 are targeted by miR-222 in liver and adipose [153]; miR-145 in liver [154]; and miR-29a and miR-29c in muscle [155]. Likewise, IRS2 expression can be suppressed by miR-126 [156], miR-33b, and miR-135a [157].

9. HETEROLOGOUS SYSTEMIC MECHANISMS OF INSULIN RESISTANCE

9.1. Introduction

Insulin resistance refers to any state in which the response to insulin (exogenous or endogenous) is lower than normal. Multiple pathologic mechanisms associated with over-nutrition and inactivity promote insulin resistance and type 2 diabetes [60,62,158—162]. In addition to hyperinsulinemia itself, insulin resistance is influenced by many factors, including age, weight, ethnicity, body fat, physical activity, dietary intake, gut microbiota, and medications [163—165]. Elevated circulating insulin concentrations can downregulate insulin receptors and desensitize post-receptor pathways [166], including reducing IRS2 expression in liver [167]. In addition, while elevated insulin works to promote glucose utilization and storage to defend against hyperglycemia, it also increases hepatic lipogenesis, leading to hyperlipidemia, WAT expansion, and hepatic steatosis [164]. This is associated with the accumulation of nutrient-derived toxic metabolites, including NEFA, DAG (diacylglycerol), and ceramides. These can activate novel PKCs (protein kinase C) to mediate Ser/Thr phosphorylation of IRS1 and InsR and thereby inhibit early steps in insulin signaling [165]. The important role of hyperinsulinemia in promoting insulin resistance is substantiated by better glucose tolerances in mice with genetically-attenuated hyperinsulinemia subjected to high-fat diets [168,169]. Nutrient excess can also increase circulating branched-chain amino acid levels, which stimulate mTORC1 that can further inhibit IR → IRS1/IRS2 signaling [170,171]. Chronic mTorc1 activity also exacerbates the ER (endoplasmic reticulum) stress response by activating an UPR (unfolded protein response) that can cause insulin resistance [172—175].

9.2. Inflammation: IL6 and TNFα

Chronic inflammation is an important cause of systemic insulin resistance. It occurs in adipose tissue, liver, pancreatic islets, vasculature, and other tissues during obesity and T2DM [163,172,176]. Expanding visceral adiposity creates a microenvironment conducive to

inflammation owing to hypoxia, adipose cell death, and dysregulated adipokines, including decreased adiponectin and increased leptin, resistin, and RBP4 [177,178] as well as accumulation of proinflammatory M1-like adipose tissue macrophages (ATMs) [179]. This contributes to increased local and circulating concentrations of proinflammatory cytokines, including MCP1 (monocyte chemoattractant protein 1), IL6 (interleukin 6), and TNFα (tumor necrosis factor-α) [180,181]. These cytokines contribute to insulin resistance by stimulating Ser/Thr phosphorylation of IRS proteins and increasing levels of SOCS proteins, which can inhibit InsR signaling [182] both directly [132,183] and through the action of JNK1 and IKKβ (Figure 6) [163,165]. Despite extensive evidence for this in murine models, clinical trials of TNFα → JNK inhibition in humans have been disappointing [165].

Activation of the innate immune response during obesity also increases the production of so-called inflammasomes and is associated with significant insulin resistance. This is mediated in part by elevated levels of fatty acids acting on Toll-like receptors, especially TLR2 and TLR4. In mice, activation of TLRs in cells results in insulin resistance, whereas genetic disruption of the TLR4 receptor in mice instead protects against fatty acid—induced insulin resistance [184].

9.3. ER stress and the unfolded protein response

The ER (endoplasmic reticulum) is a network of interconnected membrane-enclosed tubes that are continuous with the outer membrane of the nuclear envelope. The unfolded protein response (UPR) is activated when circumstances disrupt protein folding—including glucose and energy deprivation, cholesterol accumulation, viral infection, and other factors that dysregulate protein synthesis [185,186]. Hyperactivation of mTORC1 secondary to nutrient excess and chronic hyperinsulinemia promotes ER stress through increased flux of newly-synthesized proteins through the ER lumen. Three distinct branches of the UPR are initiated by two type-I transmembrane kinases, PERK (PKR-like endoplasmic reticulum kinase) and IRE1 (inositol requiring enzyme-1), and by type-II transmembrane transcription factor ATF6 [185,186]. PERK-mediated phosphorylation of eIF2 promotes lipid accumulation in the liver, which can contribute to insulin resistance [187]. IRE1 signaling activates JNK in the liver and adipose tissue to increase Ser/Thr phosphorylation of IRS1 [188]. ATF6 upregulates expression of the transcription factor XBP1, which helps resolve ER stress in obesity and insulin resistance [188]. ATF6 → XBP1 also interacts with FOXO1 to direct it toward proteasome-mediated degradation, which can contribute to systemic insulin sensitivity [189]. In addition to proteotoxic stress activation, the ATF6 detects sphingolipids and ceramides to link UPR to dysregulated lipid homeostasis [190]. Attenuation of ER stress in obese and diabetic mice by chemical chaperones attenuates insulin resistance and improves glucose tolerance [191,192]. The presence of ER stress in the liver and adipose tissues of obese patients suggests that this system plays a role in the development of obesity-linked insulin resistance [193].

9.4. Adipose tissues and insulin resistance

The association of obesity with T2DM has been recognized for decades. Central (visceral) adiposity, compared to total obesity, is more strongly linked to insulin resistance and metabolic abnormalities, including elevated plasma glucose, insulin, cholesterol, and triglyceride concentrations and decreased plasma HDL cholesterol [194]. By contrast, higher levels of subcutaneous fat may be protective against insulin resistance [195,196]. The reason for the tight link between intra-abdominal fat and metabolism is multifactorial. Abdominal fat is more lipolyticly active than subcutaneous fat, releasing more FFA into circulation [197,198]. Intra-abdominal fat also has higher levels of HSD11B1 (11β-hydroxysteroid dehydrogenase type 1), which enhances conversion of inactive cortisone to active cortisol, promoting insulin resistance. Conversely, subcutaneous fat makes and releases more adiponectin, an insulin-sensitizing adipokine.

Independent of this, when nutrient intake exceeds energy expenditure, the excess calories are stored as adipose or ectopic lipid in myocytes, hepatocytes, vascular cells, and β-cells where it can produce toxic metabolites, including DAG and ceramides. This in turn can trigger activation of PKC isoforms that promote insulin resistance [199,200]. The adipocyte itself can be adversely affected by accumulation of excess nutrients, leading to events that can have adverse consequences on the body, including increased expression of leptin, IL-6, IL-8, MCP-1 (monocyte chemoattractant protein-1), and GCSF (granulocyte colony-stimulating factor). These and other cytokines attract proinflammatory M1-macrophages, which release factors such as TNFα that may have local and systemic inflammatory effects to induce insulin resistance [201].

In addition to energy-storing WAT, humans and most other mammals have energy-burning BAT (brown adipose tissue). The content of BAT in humans is negatively correlated with age, obesity, and insulin resistance [202]. Rodents and humans also have beige or brite (brown in white) adipocytes, which appear mixed with WAT following cold or hormonal stimuli [203]. Like BAT, beige adipocytes express UCP1 (uncoupling protein 1). Higher levels of both brown and beige fat are associated with improved metabolic homeostasis and lower insulin resistance [204]. Whether this is through improved insulin-independent glucose utilization or some direct effect on insulin action remains to be determined.

9.5. Ectopic lipid accumulation

When the storage capacity of adipose tissue is exceeded, lipids accumulate in tissues such as muscle and liver, leading to insulin resistance and metabolic dysfunction. First-degree relatives of T2DM patients have an increase in intramyocellular fat, which correlates with insulin resistance [205]. This accumulation of triglyceride in muscle of obese and insulin-resistant persons is likely related to a mismatch between fatty acid uptake and oxidation. The increased lipolysis associated with obesity increases fatty acid delivery to muscle, which can activate PKC isoforms that inhibit insulin signaling [206,207]. However, increased muscle triglyceride is not always linked to insulin resistance. Indeed, exercise training, which increases insulin sensitivity, is also associated with increased muscle triglycerides [208] and increased fatty acid oxidation [209—213]. The reason for this dissociation is not completely understood, but may be related to differences in perilipin proteins associated with lipid droplets in muscle of obese and trained subjects [214].

Lipid accumulation in liver is a common feature of insulin resistance and T2DM, and when clinically significant is referred to as NAFLD (nonalcoholic fatty liver disease) [215]. However, excess lipid intake is not the only way to develop NAFLD. Feeding mice excess glucose or fructose induces metabolic pathways in liver that lead to NAFLD [216,217], indicating that a combination of excess macronutrients and decreased adipose tissue storage promotes lipid accumulation in the liver, which associates with insulin resistance.

9.6. Mitochondrial abnormalities

A decrease in oxidative capacity is seen in both humans and animals with insulin resistance, obesity, and T2DM [218,219]. Increases in intramyocellular fat content in skeletal muscle, associated with insulin resistance, may be caused by alterations in mitochondrial mass.

Expression of nuclear-encoded genes that regulate mitochondrial biogenesis and electron transport chain activity—such as PGC1α and PGC1β—is downregulated in obese patients with impaired glucose tolerance and T2DM [220–222]. The cause–effect relation between alterations in mitochondrial mass/function and skeletal muscle insulin resistance remains debated; however, an impairment of β-oxidation of fatty acids that increases even-chained acyl-carnitine levels in plasma is a marker of insulin resistance [223]. Post-translational modification of mitochondrial proteins by acetylation, succinylation, or malonylation also provides a potential mechanism for control of mitochondrial flux and insulin resistance [224]. This is controlled by levels of substrate and the sirtuin family of dacetylases. Sirt3 is the primary mitochondrial deacetylase that is activated by NAD^+ and can deacetylate critical metabolic enzymes, including ACADL (Acyl-CoA Dehydrogenase Long Chain) in liver and the pyruvate dehydrogenase complex in muscle [225,226]. Sirt3 knockout mice exhibit decreased oxygen consumption and develop oxidative stress in skeletal muscle, leading to JNK activation and impaired insulin signaling [226]. Similarly, the NAD^+-dependent Sirt5 leads to desuccinylation and demalonylation of mitochondrial enzymes, altering their activity [227,228].

9.7. Skeletal muscle

The primary site of glucose disposal after a meal is skeletal muscle, and the primary mechanism of glucose storage is through its conversion to glycogen [229]. Studies using the hyperinsulinemic-euglycemic clamp technique have demonstrated that insulin-resistant people, with or without T2DM, have a deficiency in the nonoxidative disposal of glucose. This is related primarily to a defect in glycogen synthesis, which itself is secondary to a decrease in insulin-stimulated glucose uptake [230,231]. A major question is to what extent extrinsic factors versus intrinsic factors lead to insulin resistance.

Increased fatty acid flux into skeletal muscle, related to increased visceral lipolysis, has been implicated as one of the extrinsic factors in the inhibition of muscle glucose uptake. More recent studies in humans suggest that the primary effect of fatty acids, at least in the presence of high insulin levels, is a decrease in glucose transport, as measured in vivo using ^{13}C- and ^{31}P nuclear magnetic resonance (NMR) spectroscopy [232,233]. These studies also found increased activity of novel isoforms of protein kinase C, including PKCθ and PKCδ, that might mediate the effect of elevated fatty acids to inhibit PI3K activity [206,207]. PKC-mediated serine phosphorylation of the IKKβ subunit, leading to its degradation and the unregulated translocation of NF-κB into the nucleus, may also be important to fatty acid–induced insulin resistance [234]. Disruption of the IKKβ inflammatory pathway by high-dose salicylate therapy improved insulin sensitivity in a small human trial [235].

Additionally, skeletal muscle insulin resistance may relate to changes in fatty acid and triglyceride (TG) metabolism [236]. Malonyl-CoA is an allosteric inhibitor of CPT1, the enzyme that controls the transfer of long-chain fatty acyl-CoAs into the mitochondria [237–239]. In the presence of elevated glucose and insulin levels, the TCA cycle is activated, resulting in an increase in citrate in the cytoplasm through increased mitochondrial malate cycling. The increased citrate is converted to acetyl-CoA through citrate lyase and thus provides an indirect substrate for ACC (acetyl-CoA carboxylase). Even during insulin-resistant states and T2DM, glucose uptake into skeletal muscle is higher than normal due to elevated circulating glucose and more GLUT4 at the plasma membrane owing to rising AMPK activity [240,241]. This glucose is shunted toward the glycolytic pathway, generating acetyl-CoA that can be converted to malonyl-CoA in the cytoplasm by the action of the highly-regulated enzyme ACACA [242]. This results in a buildup of long-chain acyl-CoAs and diacylglycerols, which can activate one or more PKC isoforms, leading to insulin resistance [236].

9.8. The gut microbiome and metabolome

There is growing evidence that the gut microbiome—the bacteria that reside in the gastrointestinal tract—can be a major mediator of the effect of diet in obesity, diabetes, and metabolic syndrome and can contribute to the development of insulin resistance in these disorders [243–252]. In mice, it has been shown that administration of low dose antibiotics early in life may predispose to obesity and glucose intolerance by perturbing the development of a normal microbiome [253]. The mechanisms by which gut microbiota affect pathogenesis of diabetes, obesity, and insulin resistance are complex. Gut microbiota have major effects on intestinal barrier function, breakdown of otherwise indigestible dietary components, modification of bile acids and other substances, development of the gut, and education of the immune system [243,254]. These effects can lead to a release of bacterial proteins, endotoxins, and cytokines into the bloodstream [255,256] as well as produce changes in hundreds of metabolic products, including bile acids, short-chained fatty acids, amino acids, and many other classes of molecules [246,247,257–260]. Together, these lead to tissue-specific metabolic dysregulation and immune activation, leading to insulin resistance and progression of diabetes pathogenesis. A number of metabolites have been shown to correlate with insulin resistance in both mice [261] and humans, and some, such as 2-aminoadipate, alpha-hydroxybutyrate, and N-acetylglycine, are suggested to be biomarkers of T2DM or insulin resistance [261–264]. Further studies are needed to determine exactly how gut microbiota affect insulin sensitivity and diabetes progression and whether therapies that change gut microbiota can be used to treat or prevent type 2 diabetes.

9.9. Intrinsic factors and cell-autonomous insulin resistance

It is well recognized that insulin resistance precedes and predicts type 2 diabetes, even when none of the known extrinsic factors leading to insulin resistance are present [265]. A major challenge has been identifying the intrinsic cellular defects which underlie insulin resistance and might be more intricately linked to the genetic determinants of disease. Muscle biopsies and primary cultured myoblasts derived from people with T2DM show insulin resistance, including impaired insulin signaling at the level of IRS1-associated PI3K and AKT activity and decreased glucose uptake and glycogen synthesis [222,229,266–268]. Recently, Batista et al. have used iPSC (induced pluripotential stem cell) differentiated into muscle (iMyos) to study ex vivo signaling defects in T2DM subjects' skeletal muscle in the absence of extrinsic stimuli [269]. They showed that iMyos from individuals with T2DM shows defects in insulin signaling at the level of AKT/GSK3/FOXO1 phosphorylation, decreased insulin-stimulated glucose uptake, and altered mitochondrial respiration similar to the defects observed in muscle in T2DM in vivo. Global phosphoproteomics revealed that these defects are part of a much larger multi-dimensional network of signaling changes involving over 1000 Ser/Thr phosphorylation sites on more than 700 different proteins. Only a small proportion of these abnormalities are in classical insulin-regulated phosphorylation that defines critical nodes in insulin action [100]. Indeed, the largest number of perturbations occurred in pathways outside of the canonical insulin signaling

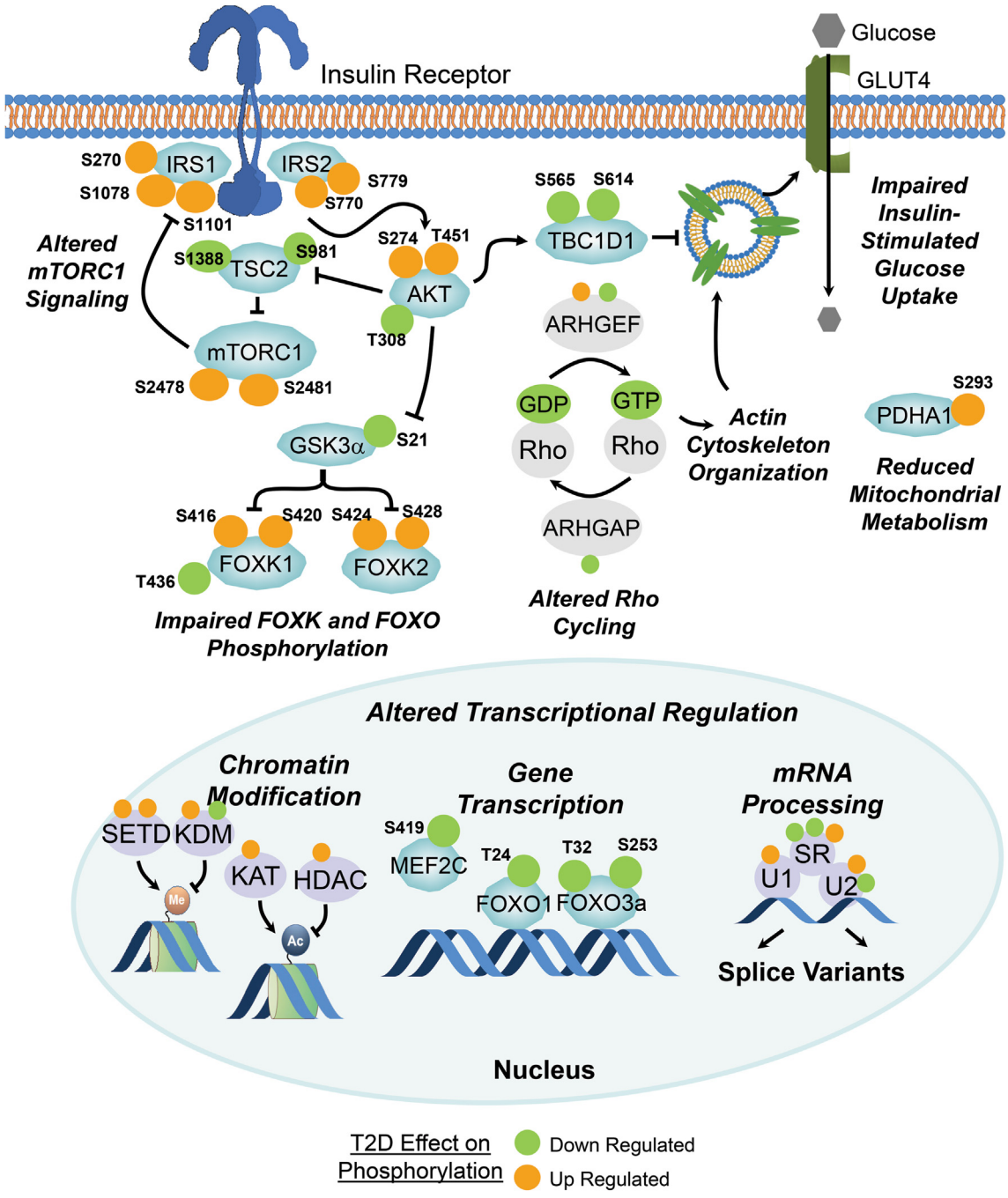

Figure 7: Schematic diagram showing some of the changes in phosphorylation observed in iPS cell—derived myoblasts from control and T2DM patients. The sites highlighted in orange are increased in either basal or stimulated phosphorylation in cells of the T2DM patients, whereas those highlighted in green are decreased in their phosphorylation. Note that many of the altered phosphorylations occur in pathways outside the pathways considered canonical insulin signaling. Figure was adapted from the data of Batista et al. [269].

pathway and not (acutely) regulated by insulin. These include up- and downregulation of phosphorylation in proteins involved in cytoskeleton remodeling, vesicle trafficking, and RHO GTPase activity and nuclear proteins involved in transcription, mRNA splicing, and chromatin remodeling (Figure 7). These findings indicate that there is a primary cellular defect underlying the insulin resistance of T2DM, and that defining what drives these defects at a molecular and cellular level will not only help in understanding the pathogenesis of type 2 diabetes but open avenues for new treatments.

10. CONCLUSIONS

Insulin and IGF-1 signaling are present in virtually every cell of the body and play a central role in the control of metabolism, growth, and differentiation. Since the discovery of the insulin receptor 50 years ago, major progress has been made in dissecting these pathways and understanding some of the many drivers of insulin resistance in T2DM, obesity, and the metabolic syndrome. These include a range of both cell-extrinsic and cell-intrinsic factors. It is clear, however, that more

remains to be learned about the integration of this systemic regulatory system, translating our understanding of these pathways into new therapies for insulin resistance—associated diseases in an important challenge for the next decade.

CONFLICT OF INTERESTS

M.F.W. is an advisory board member of Housey Pharma (https://www.housey.com/). C.R.K. is on the scientific advisory board or serves as a consultant for Kaleido Biosciences, CohBar, ERX Therapeutics, and Cellarity.

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