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Integrating adipocyte insulin signaling and metabolism in the multi-omics era

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Abstract:	Insulin-stimulates glucose uptake into adipocytes via mTORC2/AKT signaling and GLUT4 translocation and directs glucose carbons into glycolysis, glycerol and TAG synthesis, and de novo lipogenesis. Adipocyte insulin resistance is an early indicator of type 2 diabetes in obesity, a worldwide health crisis. Thus, understanding the interplay between insulin signaling and central carbon metabolism pathways that maintains adipocyte function, blood glucose levels, and metabolic homeostasis is critical. While classically viewed through the lens of individual enzyme-substrate interactions, advances in mass spectrometry are beginning to illuminate adipocyte signaling and metabolic networks on an unprecedented scale, yet this is just the tip of the iceberg. Here we review how 'omics approaches are helping to elucidate adipocyte insulin action in cellular time and space.

RESPONSE TO REVIEWERS

Reviewer 1: Insulin signaling is critical for multiple aspects of animal physiology. In adipocytes, insulin facilitates entry of glucose and de novo synthesis of lipid, and inhibits breakdown of fat, thus promoting storage of energy as fat. Because adipocyte insulin resistance has been associated with glucose intolerance, understanding the mechanism and regulation of insulin signaling in adipocytes is important. In this review, Guetin and colleagues review "Omics" approaches to study adipocyte insulin signaling pathway action in cellular time and space. The authors covered their and others' recent works and provided remaining questions in this field. Although it will be much more informative to introduce detailed modern technologies and limitations of single-or multi-Omics approaches using adipocytes, this study does go a long way in summarizing advantages of Multi-Omics approaches and our current understanding of adipocyte insulin signaling. For this reason, this study is highly significant and should be published in Trends in Biochemical Sciences. With this said, several (mostly minor) issues need to be addressed prior to publication.

Major point. Although an overview of recent omics technologies is not a focus of this review, it would be very informative to have a brief introduction of omics types and methods for their integration across multiple omics layers. In addition, it would be helpful to provide current challenges including sample preparation, and considerations for the design of omics studies.

Response: *We thank the reviewer for the comments and we have now included an additional BOX 3 that focuses on phosphoproteomics and metabolomics workflows.*

Minor points.

1. The nomenclature for protein should be in upper-case, for example, AKT not Akt.
2. In Figure 1, an arrow on top of main figure should be removed.
3. Box1 Figure legend: Abbreviations of insulin response (IR) and beta-adrenergic response (AR) should be revised, because IR and AR are more commonly used for insulin receptor and adrenergic receptor.
4. Table 1: "Genetic disruption of mTORC2"-"GLU4" should be GLUT4.

Response*: We thank the reviewer for the comments. All these minor points have been addressed in the current version of the manuscript.*

Reviewer 2: The authors introduce basic knowledge about responses to insulin in adipocytes, especially about regulation from mTORC2/AKT signaling, GLUT4 translocation and metabolites. They summarized recent advances of this topic using omics measurements. They focused on phospho-proteomics and metabolomics including flux analysis in cultured cells. They further discussed the future perspective including extension of omics measurement to in vivo studies, and revealing the cause of insulin resistance in adipose tissue.

This is a simple, very-written review for overviewing recent progresses of application of omics techniques to the research of insulin action in adipose tissues. I have only one minor comment that would improve the review.

1. In Lines 187-198, the authors discussed the relationship between the TCA cycle and DNL. Several studies suggest that time-resolved data of metabolites, fluxes, and regulatory contributions in the TCA cycle can be classified into several clusters (45, 46 and 73, the same reference numbers in the manuscript). This would provide a novel insight into the TCA cycle and the description about this in the text is appreciated

Response*: We thank the reviewer for the comments. We have now expanded our discussion on how time-resolved data has revealed insight into glucose metabolism via the TCA cycle after insulin stimulation (see lines 243-293).*

Highlights

1. Advances in proteomics and phospho-proteomics is allowing analysis of insulin signaling dynamics at unprecedented detail.

2. Advances in metabolomics and stable isotope tracing is similarly allowing analysis of carbohydrate and lipid metabolism at unprecedented detail.

3. We discuss how integrating these two 'omics techniques is critical to elucidated adipocyte insulin action in health and disease.

4. Key future directions include advancing in vivo studies and overcoming the challenge of adipose tissue heterogeneity

Integrating adipocyte insulin signaling and metabolism in the multi-omics era

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Keywords: Insulin, white and brown adipose tissue, glucose, metabolism, phosphoproteomics, metabolomics

Abstract

Insulin-stimulates glucose uptake into adipocytes *via* mTORC2/AKT signaling and GLUT4 translocation and directs glucose carbons into glycolysis, glycerol and TAG synthesis, and *de novo* lipogenesis. Adipocyte insulin resistance is an early indicator of type 2 diabetes in obesity, a worldwide health crisis. Thus, understanding the interplay between insulin signaling and central carbon metabolism pathways that maintains adipocyte function, blood glucose levels, and metabolic homeostasis is critical. While classically viewed through the lens of individual enzyme-substrate interactions, advances in mass spectrometry are beginning to illuminate adipocyte signaling and metabolic networks on an unprecedented scale, yet this is just the tip of the iceberg. Here we review how 'omics approaches are helping to elucidate adipocyte insulin action in cellular time and space.

Interplay between insulin signaling and metabolism in adipocytes

Overweight and obesity affects nearly 2 billion people worldwide. Adipose tissue expands when energy intake exceeds energy expenditure, storing the excess nutrients in lipid droplets as inert triacylglycerols (TAGs) and thereby maintaining metabolic homeostasis. However, chronic overnutrition can overrun adipose tissue's protective capacity eventually leading to obesity-associated **insulin resistance**, toxic lipid accumulation in non-adipose tissues such as liver, and a myriad of other deadly comorbidities including type 2 diabetes and cancer. Similar pathologies develop in the absence of adipose tissue (i.e. **lipodystrophy**)[1]. Thus, both adipose tissue function and amount are critical health metrics and understanding their biological basis is essential to combating the obesity epidemic.

Insulin, a peptide hormone produced by pancreatic beta cells, is a critical regulator of adipocyte metabolism. In white adipocytes [Box1], **insulin** stimulates glucose uptake and *de novo* lipogenesis while suppressing lipolysis. The key mediator of intracellular insulin action is the protein kinase AKT [Figure 1A-C]. Impaired AKT phosphorylation is often, although not always, observed in insulin resistance where insulin-stimulated glucose transport is decreased[2]. However, whether defects in insulin signaling and decreased AKT activation are a primary cause of insulin resistance, or indeed a consequence *(e.g.* of insulin resistance-induced hyperinsulinemia), remains unclear (*reviewed in [2,3]*).

Metabolites can also modulate insulin action. For example, glucose-derived metabolites have allosteric roles in glycolysis and regulation of ChREBP, a transcription factor that drives expression of glucose and lipid metabolism enzymes[4,5]. The nutrient sensing mTORC1 pathway is also regulated by the availability of glycolytic metabolites and certain amino acids[6] [Figure 1B]. Additionally, AKT and mTORC2 can be influenced by post-translational modifications regulated by metabolism such as acetylation[7] and O-GlcNAcylation[8], and the insulin-responsive glucose transporter GLUT4 can be palmitoylated to promote maximal insulin response[9]. Signaling metabolites are altered in insulin resistance raising the possibility that metabolite-signaling is also central to the aetiology of insulin resistance. Here we review recent insights into adipocyte signaling and metabolism that have been enabled, in large part, by phosphoproteomics and metabolomics approaches. We suggest that integrated assessment of signaling, metabolism and gene expression will likely shed insight into the aetiology of adipocyte insulin resistance.

The Insulin-regulated phosphoproteome in cultured adipocytes

The full insulin signaling network and its temporal and spatial dynamics remained vague until 2013 when advances in mass spectrometry provided a glimpse of the protein phosphorylation complexity of insulin signaling in adipocytes[10]. Key advances in phosphopeptide enrichment[11], sample throughput, reproducibility and accuracy (e.g. 96-well plate workflows[12], use of metabolic/isobaric labeling[11,13], and in the instrumentation and analysis software itself (*reviewed in [14]*)) allowed the description of insulin signaling with unprecedented depth in cultured 3T3-L1 adipocytes[10]. These studies revealed that approximately 20% of the adipocyte phosphoproteome is insulin-regulated within 20 minutes

post-stimulation[10,15]. In the largest study-to-date, this represents nearly 5,000 phosphorylation events on over 2,000 proteins (approximately 1/3rd dephosphorylated and 2/3rd phosphorylated[10]). This is a staggering amount of new information on the adipocyte insulin-signaling network, with the vast majority of these signaling events unstudied[14], and offers an exciting opportunity to increase our understanding of how insulin signaling modulates multiple cellular processes, including metabolism.

Phosphoproteomic analysis has also been used recently to examine insulin signaling in cultured brown adipocytes, which regulate **thermogenesis** [Box 1][16,17]. Aligning temporal signaling information from cultured 3T3-L1 and brown adipocytes reveals that the insulin response is similar between the cell types, at least in culture, and this has provided additional insights into the topology of the insulin signaling network[16]. Signaling events proximal to the plasma membrane (PM), such as insulin receptor phosphorylation and AKT activation [Figure 1A] occurred very early, within 15-30 seconds of insulin stimulation. Phosphorylation events occurring further downstream in the network (such as activation of mTORC1/S6K signaling [Figure 1B-C] and inactivation of PKA signaling, and phosphorylation events occurring at distal sub-cellular locations, such as in the nucleus), occurred later, within 2-5 minutes of insulin stimulation. This kinetic information allows novel signaling events to be more accurately mapped to specific sites within the signaling network and can be used to help predict kinase-substrate relationships. For example, AKT and S6K share the same linear consensus motif (R-X-R-X-X-S/T), but incorporating temporal information distinguishes between AKT and S6K substrates[18].

Post-translational regulation of AKT

AKT2 is the most critical AKT isoform expressed in mature adipocytes [Box 2]. Despite years of study, AKT regulation is still not fully understood. AKT is activated at the PM by PDK1 [Figure 1A] which phosphorylates AKT2 at Thr309 and mTORC2, which phosphorylates AKT2 at Ser474[19]. In some settings, these phosphorylation events are linked, while in others they can occur independently[20]. AKT phosphorylation is widely used as an intracellular biomarker of insulin resistance. Therefore, understanding the exact regulation and function of these AKT phosphorylation events is critical.

Recent phosphoproteomic approaches have shed additional light on the details of AKT and mTORC2 in adipocytes. In 3T3-L1 adipocytes, this includes identifying the mTORC2 component SIN1 as an AKT substrate (Thr86) [Figure 1A][10]. AKT signaling to SIN1 may promote mTORC2 activity by forming a positive regulatory loop. In this model, AKT (phosphorylated at Thr309) then phosphorylates SIN1 to increase mTORC2 activity towards AKT Ser474[10,21] [Figure 1A]. Doubly phosphorylated AKT is biochemically maximally activated. Other regulatory loops include feedback from mTORC1/S6K to inhibit IRS proteins (*reviewed in [6]*) and mTORC2[22,23] and a feedback loop to IRS by AKT that lowers IRS abundance at the PM[24] [Figure 1B]. These examples are likely the tip of the iceberg in understanding signaling crosstalk and highlight how unbiased approaches illuminate novel mechanisms.

Interestingly, while mTORC2 is required for maximal AKT activation, genetic models of mTORC2 loss do not phenocopy AKT loss[25,26]. For example, deleting the essential mTORC2 regulatory subunit *Rictor* does not have as strong an impact on AKT downstream signaling in white (WAT) and brown adipose tissue (BAT), as measured by more traditional approaches (e.g. immunoblotting with phospho-specific antibodies), despite profoundly impairing metabolism[17,26–29], nor does it cause adipose tissue lipoatrophy as observed when AKT1/2 are deleted[30–32].

Proteomics and phospho-proteomic analysis of insulin signaling in cultured brown adipocytes lacking *Rictor* reveal only mild effects on the global insulin response, and that only a subgroup of AKT substrates show strong mTORC2-dependency[16,33]. The top scoring mTORC2-dependent AKT substrate in this model is ATP-citrate lyase (ACLY) [Figure 1C-D], which cleaves citrate to make acetyl-CoA and oxaloacetate in the first step of *de novo* lipid and cholesterol biosynthesis[16]. This raises the possibility that some AKT substrates are more dependent on mTORC2 than others. Similar observations have been made in 3T3-L1 adipocytes using a different approach that directly inhibits AKT-S474 without impairing mTORC2[34,35] [Table 1]. However, in this model phospho-S474 loss shows ~50% AKT inhibition[16], possibly suggesting differences between cell types, experimental strategies, or compensatory mechanisms (*summarized in* [Table 1]). Nevertheless, these observations indicate that many unanswered questions exist regarding the role of mTORC2 in insulin and AKT signaling.

In summary, applying phospho-proteomics is revealing the temporal and spatial organization of the insulin signaling network. Importantly, thousands of uncharacterized insulin-regulated phosphorylation events remain unexplored. There are some limitations to current strategies, including the lack of subcellular resolution. For example, AKT may be activated at different locations in the cell, such as at the mitochondria-associated endoplasmic reticulum (ER) membranes[36] and in the nucleus[37], and whole-cell approaches cannot distinguish these or AKT activation mechanisms at these sites. The insulin dose, physiological nutrient levels in the medium, other circulating metabolites, beta-adrenergic signals, and/or communication from neighboring cells also likely impact the insulin response, and these have not yet been explored. The data discussed herein was also generated using cultured cells and translating these studies to *in vivo* insulin action is a critical next step[38,39].

Probing adipocyte metabolism using metabolomics

Insulin signaling increases glucose uptake into adipocytes through the translocation of the glucose transporter GLUT4 to the PM [Figure 1C-D]. This is thought to be largely controlled by AKT-mediated phosphorylation the Rab-GTPase activating protein TBC1D4/AS160, which then permits Rab-mediated translocation of GLUT4-containing vesicles to the cell surface (*reviewed in[40]*). In addition to stimulating glucose uptake, insulin promotes glucose incorporation into lipids and glycogen (i.e. *de novo* lipogenesis and glycogen synthesis), free fatty acid uptake, and glycerol and TAG synthesis while simultaneously suppressing lipolysis [Figure 1C-D]. This promotes the safe storage of lipids in TAG droplets. Adipose tissues both maintain an abundant energy reserve and protect the body against toxic lipid accumulation in

non-adipose tissues such as the liver [Box 1][41,42]. Although these basic tenets are well established, how individual metabolic pathways and intermediary metabolites function in normal and disease states is not well understood, especially *in vivo*.

Glucose carbons as well as the carbon and nitrogen components of other circulating nutrients like amino acids and short and long chain fatty acids can be used in energy storage pathways, as building blocks for organelles, proteins, and nucleic acids, and as signaling and allosteric regulatory molecules. As an analytical profiling technique, metabolomics can identify and quantitate the metabolites present in a biological sample thereby providing a snapshot of a cell's or tissue's metabolic state. However, this only gives part of the picture because it does not reflect the consumption or production rates of individual metabolites. For this, **stable isotope tracing** (e.g. with ^{13}C-labeled or other compounds) allows substrate metabolism to be followed over time providing a measure of the flow rate (or flux) of individual carbons through metabolic pathways as well as their destination[43].

Stable Isotope Tracing in 3T3-L1 adipocytes

Stable isotope tracing with ^{13}C-glucose has been performed in 3T3-L1 adipocytes[44]. These studies suggest glucose is rapidly processed via glycolysis and also the pentose phosphate pathway (PPP)[44]. Temporal analysis of insulin-stimulated protein phosphorylation versus changes in glucose metabolism reveals that many insulin-regulated phosphorylation events on metabolic enzymes occur prior to increased glucose transport, and that lower glycolysis increases flux before upper glycolysis[45]. The implications are that insulin signaling prepares, or primes, metabolic pathways ahead of the glucose deluge by acting at multiple points beyond regulating GLUT4. This priming may drag glucose towards specific metabolic routes such as the PPP (to make NADPH for lipid synthesis)[44], the glycerol-3-phosphate (to make the backbone for TAG synthesis)[46], and *de novo* lipogenesis (DNL) pathway (discussed below).

An interesting aspect of these and follow-up studies in 3T3-L1 cells[44,45,47] is that glucose-carbon flux through glycolysis and into the tricarboxylic acid (TCA) cycle can be classified into distinct metabolic regulatory clusters. For example, insulin increases the concentration of upper glycolysis metabolites to reach a new steady state within the first 10 minutes of stimulation, while lower glycolysis metabolites take nearly 4 times longer to reach equilibrium[44]. Nevertheless, glycolysis metabolites overall cluster together increasing in abundance with time, and in a manner in which glycolysis operates faster than the TCA cycle (evidenced by increased lactate production). Indeed, evidence from both adipose tissue and cultured adipocytes suggests that a substantial portion of glucose taken into the cell in response to insulin is converted to lactate[44,48,49], though the proportion may vary depending upon cell type (3T3-L1 versus primary adipocyte) and/or culture conditions[49]. Why insulin more strongly drives glycolytic flux than TCA cycle flux is unclear. Converting pyruvate to lactate via lactate dehydrogenase may be important to regenerate the NAD^+ consumed during glycolysis. Increased glycolytic flux could also reflect an advantage for increased ATP generation by aerobic glycolysis, increased flux into the PPP for NADPH synthesis, increased carbon demand for synthesizing glycerol (to make TAGs), or a combination of these factors.

Pyruvate carbons derived from glucose can also enter the TCA cycle after conversion to acetyl-CoA and then citrate. Compared to glycolytic metabolites, the kinetics and extent of glucose-derived carbon enrichment in TCA cycle metabolites following insulin stimulation is more heterogeneous[44]. Malate and fumarate appear to cluster with glycolysis metabolites and have a higher total increase while citrate, isocitrate, and succinate levels initially drop prior to increasing but to an overall lower amount. The distinct clustering of these TCA metabolites, along with the isotopologue distributions (i.e. the patterns of labeled carbons within TCA metabolites) suggest glucose flux via the pyruvate anaplerosis pathway[44] [Figure 1D]. The initial decrease in citrate may also provide evidence supporting the priming effect of insulin. In anabolic conditions, citrate molecules are exported from the mitochondria for entry into the DNL pathway. Extra-mitochondrial citrate is cleaved by ATP-citrate lyase (ACLY) to form acetyl-CoA and oxaloacetate, and ACLY is regulated by insulin through post-translational and allosteric mechanisms (discussed below).

Acetyl-CoA is further carboxylated to form malonyl-CoA by acetyl-CoA carboxylase (ACC) committing it to lipid synthesis, and then assembled into lipids by fatty acid synthase (FASN) in the rate limiting step of DNL [Figure 1D]. Acetyl-CoA can also enter the mevalonate pathway leading to cholesterol and isoprenoid biosynthesis although this pathway has been less studied in adipocytes. The cytoplasmic oxaloacetate generated by ACLY is converted to malate, which can be decarboxylated to pyruvate by malic enzyme (ME) in the cytoplasm in an NADPH generating reaction or shuttled back into the mitochondria [Figure 1D]. Interestingly, cytoplasmic ME1 appears to be a main source of NADPH in certain models suggesting that pyruvate regeneration could be required for DNL[46,50]. Tracer experiments suggest that pyruvate can be converted to both oxaloacetate by pyruvate carboxylase, which is activated within 5 min of insulin stimulation[44], and acetyl-CoA via PDH, thereby allowing glucose to potentially operate as the sole or at least major carbon source for citrate production under insulin stimulation.

Despite this key role for glucose in lipogenesis, the contribution of malonyl-CoA from non-glucose substrates is substantial and maintained independent of insulin status[46]. As indicated above, exporting citrate for DNL depletes TCA cycle intermediates and maintaining TCA cycle flux requires engagement of **anerplerotic pathways**, which can then contribute to acetyl-CoA for lipogenesis. Alternate carbon sources include branched-chain amino acids (BCAAs)[51,52], glutamine[50] and acetate[17,53,54]. In fact, BCAAs account for approximately 30% of the lipogenic acetyl-coA in 3T3-L1 adipocytes[52] consistent with adipose tissue being a key site of BCAA metabolism[55]. Thus, while the emerging picture is one of inherent substrate flexibility, much more work is needed especially in vivo to establish the physiological relevance of glucose versus non-glucose substrates to adipose tissue metabolism.

Insulin signaling to metabolism in 3T3-L1 adipocytes

These above combined data support the notion that one of insulin's main functions is to coordinate the synthesis of lipids and their storage from glucose. It does so by targeting multiple steps beyond stimulating GLUT4 translocation to the plasma membrane, thereby priming certain metabolic pathways to establish a model of substrate-demand-driven metabolic fluxes. Apart for stimulating GLUT4 translocation, one of the earliest effectors linking insulin to glucose

metabolism *via* AKT was the glycogen synthase kinase (GSK) and glycogen synthase (GS), although glycogen is not thought to be a major site for glucose storage in adipocytes. AKT phosphorylates and inactivates GSK3, which leads to decreased phosphorylation of GS at several sites (Ser641, Ser645, Ser649 and Ser653) [Figure 1C]. Dephosphorylation, along with increasing concentrations of its allosteric activator glucose-6-phosphate, fully activates GS and re-routes glucose into glycogen[56].

Phosphoproteomic analysis in 3T3-L1 cells identified other potential substrates linking AKT signaling to metabolism including NADK, ABDH15, SLC1A3, and PFKFB3[10] [Figure 1C-D]. Ser44 on NAD kinase (NADK) was identified as a high confidence AKT substrate by phosphoproteomic profiling in adipocytes[10]. NADK (Ser44/46/48) has now been described as a *bona fide* AKT substrate linking insulin stimulation to NADK-dependent increase in cellular NADP[57]. Stimulation of cytoplasmic NADK by insulin may increase NADPH production to support adipocyte *de novo* lipogenesis, but this has yet to be formally tested.

ABHD15 also undergoes insulin-dependent phosphorylation at several sites, including Thr142, Ser 146, Ser 304, Thr424, Ser 425, and Ser442[10]. ABHD15 is an α/β-hydrolase that interacts with PDE3B, and ABHD15 depletion impaired the suppression of PKA signaling and anti-lipolytic effects of insulin[58]. The role that ABHD15 phosphorylation plays in the regulation of lipolysis by insulin remains to be understood.

SLC1A3 is a PM aspartate/glutamate transporter that undergoes insulin-dependent tyrosine phosphorylation at Tyr523[10]. Phosphorylation does not appear to alter transport activity or localization leaving its exact function unknown[59]. PFKFB3 catalyzes the formation of fructose-2,6-BP to allosterically activate PFK1, a rate-limiting step of glycolysis. PFKFB3 is one of the most highly regulated insulin-dependent phosphoproteins in adipocytes (Ser461, Ser467;[10]). Phosphorylation reportedly increases PFKFB3 activity to promote glycolysis[60] and it is tempting to speculate that PFKFB3 phosphorylation acts in concert with other regulatory mechanisms such as insulin-stimulated HK2[36,61] and PFK2[62] activity, although this has not been studied in adipocytes. Interestingly, PFK2 was also identified as an insulin stimulated phosphorylation substrate uniquely sensitive to mTORC2 suggesting it may be co-regulated with phospho-ACLY to coordinate glucose to lipid fluxes[17]. These observations provide a glimpse at how global phosphoproteomics is revealing mechanisms linking insulin to metabolic control. Going forward it will be critical to extend these studies to primary cells, *in vivo* models, and other types of adipocytes.

A key question relating to the regulation of adipocyte metabolism by insulin signaling is the relative contribution of these phosphorylation sites and other regulatory mechanisms like allostery. To address this, Ohno *et al.* integrated data from time-resolved phospho-proteomics and metabolomics experiments to study the major regulatory mechanisms in adipocyte glucose metabolism (termed trans-omics network analysis)[47]. Their model predicted that TBC1D4/AS160-S595 phosphorylation is the sole phosphorylation event critical for insulin-regulated glucose metabolism (through GLUT4 translocation), that *DNL* is regulated by allosteric mechanisms acting in part on ACLY, and that most glycolytic reactions are regulated by the amounts of substrates and products. The authors suggest that insulin only regulates a

few key steps of glucose metabolism, namely glucose uptake. In contrast, direct experimental analysis of protein phosphorylation suggests a key role for insulin-stimulated ACLY phosphorylation in regulating ACLY activity in brown adipocytes, particularly during differentiation[17]. Future studies will need to refine these models in the context of primary cells, *in vivo* modeling, and adipocyte heterogeneity.

Metabolic Flux in Brown Adipocytes

Understanding metabolic fluxes in brown adipose tissue (BAT) is of interest given its therapeutic potential as a nutrient sink (Box 1). The major brown fat fuels are lipids and glucose and measuring glucose uptake by FDG-PET-CT is currently the best indicator of brown fat in humans[63]. Brown adipocytes are highly insulin sensitive cells, but they also respond to norepinephrine (NE) released by the sympathetic nervous system following cold exposure. Insulin and NE/beta-adrenergic signaling typically work opposite each other; for example, insulin signaling is active during anabolic states such as after feeding, while NE is critical during fasting. However, this relationship is more complex in highly innervated BAT in which anabolic and catabolic metabolism cooperate to drive the energy demanding process of adaptive thermogenesis[31,64,65].

During cold adaptation, uptake of glucose and lipids into BAT increases substantially[65]. Active brown adipocytes consume relatively large amounts of glucose and fatty acids, but exactly how these substrates are utilized varies with the degree and duration of cold exposure[31,65]. Although studies to-date have not focused on insulin, metabolomics and stable isotope tracing studies are revealing insight into BAT glucose fluxes upon cold adaptation[65]. Moreover, recent reports suggest that oxidation of TCA intermediates such as succinate can increase reactive oxygen species that may stimulate UCP1 activity[66]. BAT can also store glucose as glycogen and lipids in TAG lipid droplets, which are rapidly accessible during acute cold exposure.

The extent to which glucose is oxidized by the TCA cycle in BAT has been unclear. Recent *in vivo* isotope tracing studies with ^{13}C-glucose in mice reveal that a significant portion of the glucose taken up by cold adapted BAT has additional functions beyond directly fueling the TCA cycle. For example, during cold adaptation glucose flows into the PPP and DNL pathways, the former possibly providing NADPH to support lipid synthesis[65,67]. Glucose also rapidly fluxes (within 15 minutes of glucose uptake) into acetyl-carnitines, which are the precursors to lipid oxidation, suggesting the *de novo* synthesized lipids may be rapidly rerouted to the mitochondria and oxidized[65,68–70]. These studies highlight a less-appreciated feature of adaptive thermogenesis in which cold stimulation simultaneously activates lipid synthesis and oxidation[65]. Why this futile anabolic-catabolic lipid cycle occurs is not known.

How insulin signaling contributes to BAT metabolic fluxes may also vary with the degree and duration of cold exposure. For example, AKT phosphorylation is higher in the BAT of *ad libitum* fed mice living in a mild cold environment (22°C) compared to thermoneutrality (30°C). However, adaptation to colder temperatures (6°C) lowers BAT AKT phosphorylation despite increasing glucose uptake[25,31,65]. This may reflect more rapid nutrient consumption and thus

a lowering of circulating insulin under these conditions. Transcriptomics analysis further shows that colder temperatures result in higher expression of genes that encode glycolysis regulators suggesting transcriptional regulation of glucose metabolism may become more important during cold adaptation. Glucose uptake in brown fat is also additionally driven by the GLUT1 transporter, which is responsive to both beta-adrenergic and insulin stimuli[71], although GLUT1 is less insulin-responsive than GLUT4[34]. The interplay between signaling pathways and metabolic fluxes in BAT, and the full spectrum of BAT metabolic substrates and their intracellular fates, is just beginning to be explored.

Integrating hormone and metabolite signaling

When considering insulin action from a systems perspective, one frontier is deciphering the interplay between hormone signaling and metabolic fluxes, both acutely in healthy individuals, such as in response to a rise in blood glucose levels, and in states of chronic overnutrition like obesity and insulin resistance, in which epigenetic and transcriptomic changes can alter a cells metabolic flexibility. Within this context, one goal is to identify the key nodes that can directly respond to both insulin-signaling and metabolite availability (nutrient sensing).

One critical node linking hormone, metabolic, and transcriptional events is ACLY and its product acetyl-CoA. Acetyl-CoA is both a lipid and cholesterol precursor, but also the substrate for protein acetylation, which can regulate enzyme activity and most famously epigenetic control of gene expression. ACLY is the main source of nuclear-cytoplasmic acetyl-CoA especially from glucose and it is also an early target of insulin-stimulated AKT phosphorylation (at Ser455)[17,53,72] [Figure 1D and 2]. ACLY can also be allosterically regulated by phosphorylated sugars such as fructose 6-P[73] and this may play a role in ACLY activation following insulin stimulation[47]. Moreover, ACLY phosphorylation by AKT-mTORC2 signaling in brown adipocytes not only promotes acetyl-CoA availability and de *novo* lipogenesis, but also transcriptional expression of glucose transporters and DNL enzymes. During brown adipocyte differentiation, this is essential for establishing the epigenetic state required for induction of PPAR-gamma and the brown adipogenic program[17]. In primary subcutaneous white preadipocytes, this circuit is not required for PPAR-gamma induction, but it is required for establishing the transcriptional program for lipid synthesis[26], thus there may be some tissue-specific differences regarding development.

The insulin signaling pathway also directly targets kinases with roles in energy or nutrient sensing, such as AMP-dependent kinase (AMPK) and mTORC1. AMPK and mTORC1 are often reciprocally active, with insulin signaling promoting mTORC1 activity [Figure 1B-C, Figure 2]. AMPK is activated under times of energetic stress, when AMP levels rise (*reviewed in[74]*) and acts to attenuate energy-consuming processes such as DNL while promoting energy-producing processes like β-oxidation. The role of AMPK activity in adipose tissue biology and metabolism is not as clearly defined as in other tissues like muscle[75], although recent studies have implicated AMPK in promoting BAT-mediated thermogenesis and the browning of WAT[76]. In terms of insulin signaling to AMPK, there is substantial crosstalk between AMPK and the insulin-activated kinases AKT and mTORC1, where AKT[77] and mTORC1-mediated[78] phosphorylation of AMPK lowers AMPK activity. Further, since insulin signaling is

most active postprandially, metabolic signaling to AMPK via the AMP/ADP:ATP ratio or decreased lipolysis derived fatty acids[79] would be expected to decrease AMPK activity. Together, these mechanisms lead to lower AMPK activity when insulin signaling is active.

mTORC1 integrates metabolite and insulin signaling [Figure 2]. Moreover, most of the insulin-stimulated phosphoproteome is mTOR-dependent in mouse embryonic fibroblasts (MEFs) and Human embryonic kidney (HEK)-293E cells[80,81]. mTORC1 activity is controlled via the heterotrimeric TSC complex and two distinct small GTPases, the Rag GTPases that regulate mTORC1 subcellular localization, and Rheb GTPases that control mTORC1 kinase activity. AKT phosphorylates the mTORC1 component PRAS40 and promotes Rheb activity through TSC (*explained in detail in[82]*). Through a distinct pathway, mTORC1 is regulated by the intracellular availability of the amino acids leucine, arginine, methionine[83,84] as well as the glycolytic intermediate dihydroxyacetone phosphate (DHAP)[85] [Figure 1B], which act through the Rag GTPases. AMPK phosphorylates the TSC complex at sites distinct from AKT and stimulates its GAP activity towards Rheb to inhibit mTORC1[86].

Knockout of *Raptor*, an essential mTORC1 subunit, in adipose tissue causes lipodystrophy consistent with mTORC1 regulating adipocyte lipid storage capacity[87,88]. mTORC1 is also reported to regulate DNL through SREBP1[19,89]. Although the role of amino acids as mTORC1 signals is well established in cultured cell models, the significance of amino acid sensing in adipose tissues is not understood. This warrants investigation given the high use of branched chain amino acids in adipocytes for DNL, the strong link between high circulating branched chain amino acids (e.g., leucine, a mTORC1 regulation) and insulin resistance in obesity[90].

Other examples of signaling metabolites that are likely important in adipocytes (though currently understudied) include how ATP and long chain fatty acyl-CoA inhibits lactate dehydrogenase A[91] and how glucose-derived metabolites activate CHREBP, a transcriptional activator of the DNL program [Figure 2]. There may also be a link between glycolysis/PPP activity and insulin signal transduction since the PPP by-product γ-6-Phosphogluconolactone modulates phosphatase activity[92] and inhibition of glycolysis (by targeting PFKFB3) blunted insulin signaling[93]. The significance of these protein modifications or PPP/glycolysis-insulin signaling crosstalk in the regulation of adipocyte signaling and metabolism also remain unclear. Covalent post translational modifications of proteins by metabolites that may be linked to changes in cellular metabolism, such as acetylation[94], O-GlcNacylation[95], and glycosylation[96], are also gaining attention due to their potential to modify enzymatic activity and link substrate availability to metabolic changes. Thus, integrating post-translational modification mapping data beyond phosphorylation with **transcriptomics** and metabolic flux studies will be required to fully understand how metabolism is regulated coordinately by hormones and nutrient availability.

Concluding remarks

Combining phosphoproteomics and metabolomics, as well as transcriptomics is a powerful way to understand how adipocytes sense and respond to blood glucose and insulin

levels. To date, much of the work is cell culture-based, and while cell culture models are revealing novel regulatory mechanisms, *in vivo* studies will be required to understand physiological relevance. Although translating phosphoproteomics and metabolomics to *in vivo* models presents additional challenges, the approach is increasing in feasibility. For example, phosphoproteomic workflows have already yielded deep coverage of insulin[97] or exercise[98] signaling networks in the liver and muscle, respectively, and there are increasing examples of studies utilizing tracers *in vivo* to study glucose and amino acid metabolism[65]. Additional technologies are now coming online that may allow determination of spatial regulation of the proteome[99], phosphoproteome[100] and metabolome[101], and at the single cell level at least for some technologies[102]. MADLI-MS imaging is one exciting example in metabolomics[103]. Applying these techniques in both genetic and disease models, in combination with emerging single cell technologies, is an exciting prospect set to unravel adipocyte insulin signaling and metabolic regulation on unprecedented levels (see also Outstanding Questions).

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BOX1 *Types of adipocytes*

White adipocytes function as long term energy storage depots and critical endocrine organs (*reviewed in[104]*, Box 1 Figure 1, left). White adipose tissues (WATs) are typically classified as being subcutaneous white adipose tissue (SWAT) or visceral white adipose tissue (VWAT), the abundance of which can vary widely between individuals and populations. Importantly, SWAT and VWAT are not metabolically equivalent as excess VWAT is typically associated with poor metabolic outcomes while excess SWAT is often correlated with better health[105,106]. Brown adipocytes are functionally and morphologically different adipocytes that exist in anatomically distinct brown adipose tissue (BAT) depots [Box 1 Figure 1, right]. One of their most important functions is thermogenesis. The abundance of BAT in humans positively associates with metabolic health and resistance to obesity[63,107]. Active brown adipocytes contain multilocular lipid droplets, more mitochondria than white adipocytes, and express uncoupling protein-1 (UCP-1), which drives non-shivering thermogenesis (NST) by dissipating chemical energy as heat. Some white adipocytes can also adopt brown adipocyte characteristics under certain conditions[108] [Box 1 Figure 1, middle]. These brown-in-white (brite), inducible brown, or beige adipocytes mainly appear in subcutaneous fat depots in mice (but also in some visceral depots such as the peri-renal WAT)[30,109,110], express UCP1, and can contribute to whole body energy expenditure. The formation of brite/beige adipocytes, also called the browning of WAT, can occur with prolonged cold exposure or other metabolic stresses such as in cancer cachexia[111] or following severe burns[112] suggesting additional or alternative roles for these cells.

BOX 2 *AKT isoforms activation, substrate specificity and selective insulin resistance.*

AKT exists in three isoforms encoded by three different genes. Knockout studies suggest that AKT1 plays a major role in cell growth and survival, while AKT2 functions primarily in metabolism, and AKT3 acts in the brain/ central nervous system (CNS). Although AKT1 plays a key role in adipocyte differentiation, AKT2 is the predominant isoform in mature adipocytes and is the major mediator of AKT signaling. Each isoform undergoes phosphorylation-mediated activation at a threonine within the T-loop and HM domain. These sites are T308 and S473 in AKT1, T309 and S474 in AKT2 and T305 and S472 in AKT3. Phosphorylation of the T308 site is required for AKT activation[34,35,113–115], but the role of phosphorylation of S473 in the hydrophobic motif in the c-terminus in AKT kinase activation has been the subject of several recent studies (**Table 1**).

Selective insulin resistance, a pathophysiological state where certain aspects of insulin signaling are impaired more than others has been described in adipocytes[2]. What might explain this selectivity? One possibility is that insulin-regulated processes are differentially sensitive to insulin or AKT activation[116,117]. Although the phosphorylation status of AKT may regulate AKT substrate specificity (**Table 1**), AKT subcellular localization[118] can also affect substrate interactions. One possibility is that the selectivity of insulin resistance may result from the cellular conditions conspiring to discourage AKT activity towards specific substrates or acting at least initially on selective downstream pathways like GLUT4 translocation or ACLY activity rather than on AKT itself. However, the degree of selectivity in insulin/AKT signaling in insulin resistance is not yet understood, in part because there are no global data on insulin signaling in insulin resistance adipocytes or in adipose tissue.

BOX 3 *Considerations for using omic's approaches.*

Phosphoproteomics

Phosphoproteomics is concerned with identifying and quantifying protein phosphorylation at a global scale. Such unbiased approaches have revealed the remarkable extent of the phosphoproteome and insight into insulin signaling dynamics [10]. Phosphoproteomics encompasses 1) solubilization of proteins within a sample; 2) removal of contaminants such as detergents or lipids (e.g. protein precipitation); 3) proteolytic digestion (typically with trypsin and Lys-C); 4) phosphopeptide enrichment (e.g. using titanium dioxide or antibodies); 5) peptide desalting; 6) analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS); 7) database searching and data analysis (see [12]). The output is typically a list of phosphopeptides with information on the exact site of phosphorylation within the peptide as well abundance information which will depend on the quantification strategies used in the study (i.e. label-free quantification (LFQ), metabolic labeling, isobaric tags)[119]. Each of these steps will require optimizing for the specific sample being analyzed. For example, removal of lipids is an important consideration for studies in adipose tissue.

Phosphoproteomic study design often includes 'unstimulated' (e.g. saline) and 'stimulated' (e.g. insulin) arms because these analyses generally aim to understand how cells and tissues respond to acute stimulus (e.g. insulin). Protein phosphorylation is highly dynamic, so samples need to be snap frozen or processed within seconds to ensure protein phosphorylation stability. Phosphorylation is also sensitive to stress and time of day and all of these variables should be as tightly controlled as possible.

Metabolomics

The main tool used in metabolomics is a mass spectrometer, with chromatographic separation usually via Liquid Chromatography (LC-MS) or Gas Chromatography (GC-MS). The mass-to-charge ratio (m/z) of a metabolite combined with retention time on the column and signal intensity is used to infer the metabolite's identity and abundance. Metabolomics strategies can be targeted, in which specific metabolites are studied usually in comparison to a standard, or non-targeted/unbiased[120]. In addition to choosing the appropriate profiling strategies, careful consideration should be given to diet or nutrient conditions and to rapidly quenching metabolic reactions during cell/tissue harvest.

Metabolomics workflows can provide a metabolic profile in a sample, which is a snapshot of the amounts of different metabolites at a single time point or condition. To study pathway activity or metabolic flux, stable isotope tracing must be used. Stable isotope tracing substitutes a tracer metabolite labeled with a rare stable isotope such as carbon-13 instead of the more abundant carbon-12 isotope [121]. By sampling at different time points after administering the tracer, one can infer the rate at which the tracer is metabolized in a pathway by measuring the incorporation and distribution of carbon-13 isotopes.

Figure Legends

Figure 1. ***Insulin signaling via AKT and sites of metabolic regulation by insulin signaling in adipocytes.*** **A**, Schematic of full AKT activation via a positive feedback loop involving AKT-mediated activation of mTORC2. Canonical AKT activation occurs when PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate phosphatidylinositol (3,4,5) trisphosphate (PIP_3) at the plasma membrane (PM), to which AKT binds via its pleckstrin homology (PH) domain. This co-localizes AKT with its upstream activators PDK1 and mTORC2 **B**, Three reported negative feedback loops control AKT activation by targeting upstream signaling intermediates. **C**, Insulin signalling targets a number for cellular processes, including metabolic processes such as lipid and glucose metabolism, via AKT and its substrates (DNL; *de novo* lipogenesis). **D**, Schematic of insulin signaling to glucose and lipid metabolism. Insulin-stimulated glucose transport is increased by the delivery of the glucose transporter GLUT4 to the PM. Metabolic enzymes and transporters that undergo insulin-regulated changes in phosphorylation are highlighted (yellow star); these may act as key integration points of signaling and metabolism.

Figure 2. ***Crosstalk between signaling, metabolism and gene expression in adipocytes.*** **A,** AKT-mediated phosphorylation of TSC2 and PRAS40 activates mTORC1 in the, AKT-mediated phosphorylation of TSC2 and PRAS40 activates mTORC1 in the presence of amino acids. mTORC1 signals to activate the transcription factor SREBP1. **B**, AKT-mediated phosphorylation of ACLY leads to increased cytosolic acetyl-CoA for de novo lipogenesis (DNL). Cytosolic acetyl-CoA is used for acetyl-Lysine synthesis as a substrate for histone acetylation. **C,** AKT-mediated phosphorylation of TBC1D4 is required for GLUT4 translocation, increasing glucose uptake. Increased glycolytic and TBC1D4 is required for GLUT4 translocation, increasing glucose uptake. Increased glycolytic and pentose phosphate pathway (PPP) intermediates bind and activate the transcription factor ChREBP.

BOX 1 Figure: ***Morphological and functional differences between adipocyte cells.***
Comparison of the morphological and functions differences between adipocyte subtypes. Abbreviations: Uncoupling protein 1 (UCP1), de novo lipogenesis (DNL), insulin response (Ins. Resp.), beta-adrenergic response (Adren. Resp.).

Table 1. Studies of AKT-S473 phosphorylation in relevant to adipocyte AKT activity

Model	***Author, Year***	***Model System***	***AKT isoform***	***Method for preventing AKT 473/474 phosphorylation***	***Method(s) for assessing AKT activity?***	***Key findings regarding role of S473 in AKT activity***	***Pros and cons of approach***
IN VITRO	*Alessi, 1996[114]*	*Overexpressed AKT; HEK cells; insulin and IGF1 stimulation*	*AKT1*	*S473-->A473*	*Immunoprecipitated AKT, in vitro kinase assay - GSK peptide*	*S473A lowered AKT activity by ~90%*	Highly controlled conditions; may not replicate intracellular environment
	Balasuriya, 2018 and 2020[113, 122]	*Recombinant AKT*	*AKT1*	*Phosphorylation of AKT1 by PDPK1 at T308 in vitro*	*In vitro kinase assay - GSK peptide (2018) or AKT substrate peptides (2020)*	*AKT1 pT308, pS473 ~ 3.5-fold more active than AKT1 pT308.* *NOTE: Loss of pS473 did not affect all AKT substrate peptides similarly.*	
	Chu, 2020[123]	*Recombinant AKT (semisynthetic)*	*AKT1*	*Phosphorylation of AKT1 by PDPK1 at T308 in vitro*	*In vitro kinase assay - GSK peptide*	*Kcat for AKT1 pT308, pS473 ~ 19-fold greater than AKT1 pT308*	

Chemical-genetics / MK-resistant AKT	*Beg, 2017[34]*	*MK-resistant (W80A) AKT2 overexpression; 3T3-L1 adipocytes; insulin stimulation*	*AKT2*	*MK-resistant AKT2 WT and S474A. Expressed in fibroblasts prior to differentiation into adipocytes*	*Insulin-stimulated GLUT4, GLUT1 and TfR translocation*	*Insulin-stimulated GLUT1 translocation to the PM sensitive to loss of AKT2 pS474. GLUT4 and TfR unaffected.*	Assesses effect of specific AKT mutants in cells without interference from endogenous AKT; MK2206 may have off-target effects; long term expression of AKT constructs may lead to adaptive responses in cells
	Kearney, 2019[35]	*MK-resistant (W80A) AKT2 overexpression; 3T3-L1 adipocytes; insulin stimulation*	*AKT2*	*MK-resistant AKT2 WT and S474A. Expressed in fibroblasts prior to differentiation into adipocytes*	*Insulin-stimulated AKT substrate phosphorylation (Western blot), FOXO nuclear exclusion, protein synthesis, GLUT4 translocation/ glucose transport and GLUT1 translocation.*	*Preventing pS474 lowered all responses by ~50%, except insulin-stimulated GLUT1 translocation which was more impaired.*	
Genetic disruption of mTORC2	*Kumar, 2010[29]*	*Tissue specific KO mice - adipocyte rictor KO, aP2-cre*	*Not specific - AKT2 is predominant isoform in adipocytes.*	*Rictor KO in adipose tissue*	*Insulin-stimulated AKT substrate phosphorylation (Western blot), glucose transport, GLUT4 translocation and anti-lipolysis*	*No effect on GSK3 phosphorylation, but impaired insulin-stimulated phosphorylation of TBC1D4 and FOXO3A* *glucose uptake, GLUT4 translocation and anti-lipolysis.*	Specific analysis of role of mTORC2 in AKT signaling; ablation of mTORC2 prevents phosphorylation at other mTORC2 sites such as T450 (reportedly involved in AKT stability)[124]; may not be specific to S473 as

Tang, 2016[27]	*Tissue specific KO mice - adipocyte rictor KO, AdipoQ-cre*		*Rictor KO in adipose tissue*	*Insulin-stimulated AKT substrate phosphorylation (Western blot)*	*Specific impairment in p-AKT1S1/ PRAS40 in response to insulin. Other AKT substrates tested not affected by Rictor loss.*	mTORC2 reported to phosphorylate alternate C-terminal residues which have been shown to activate AKT[123]; long term loss of mTORC2 may lead to compensatory mechanisms
Martinez-Calejman, 2020[17]	*Inducible Rictor KO precursor brown adipocytes*		*Rictor KO in precursor and mature brown adipocytes*	*Insulin-stimulated AKT substrate phosphorylation (targeted phosphoproteomics and Western blot)*	*ACYL (S455), PFKFB2 (S486, S489) and BAD (S155) sensitive to Rictor loss. Other AKT substrates tested not affected.*	
Entwisle, 2020[16]	*MEFs differentiated into brown adipocytes*		*Induced Rictor KO after adipocyte differentiation*	*Insulin-stimulated AKT substrate phosphorylation (phosphoproteomics time course)*	*Global dampening of insulin signaling. Specific insulin-regulated phosphosites more sensitive to Rictor loss than others (e.g. ACLY).*	

Glossary

Insulin: Peptide hormone secreted by pancreatic beta cells in response to elevated blood glucose. Activates signal transduction in target tissues via binding to the insulin receptor.

Insulin resistance: impaired response of one or more tissues to normal concentrations of insulin. Results in elevated insulin requirements to maintain glycaemia.

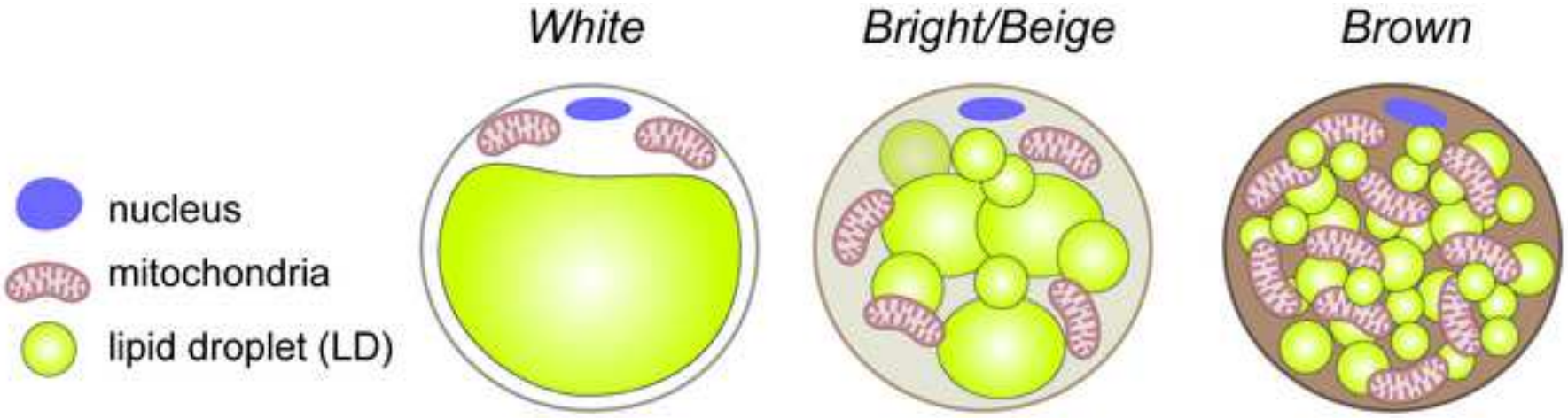
Stable isotope tracing: method for quantifying the metabolism of specific metabolites. This method uses metabolites that have one or more stable isotopes with a different mass to the most abundant natural occurring isotopes (e.g. ^{13}C, ^{15}N, ^{18}O) incorporated.

Anerplerotic pathways: metabolic pathways that replenish TCA cycle intermediates.

Transcriptomics: study of the sum of all RNA transcripts in sample (e.g. tissue) under specific physiological conditions.

Lipodystrophy: Condition characterized by a lack of, or dysfunctional, adipose tissue.

Thermogenesis: dissipation of energy through heat production. In adipocytes expressing UCP1 (e.g. brown or beige adipocytes), oxidative phosphorylation is uncoupled from ATP production leading to heat production.



Primary Functions	Energy storage Glucose Clearance Endocrine	Energy Storage? Endocrine? Thermogenesis?	Thermogenesis Endocrine Glucose Regulation?
UCP1	No	Yes	Yes
Mitochondrial density	Low	Medium	High
LD morphology	Unilocular	Multilocular	Multilocular
Glucose transporter	GLUT4 (Ins. Resp.)	?	GLUT1 (Adren. Resp.) GLUT4 (Ins. Resp.)
DNL	Higher in SWAT than VWAT	?	Increases with cold adaptation

Figure 1

Click here to access/download;Figure;Figure 1.tif

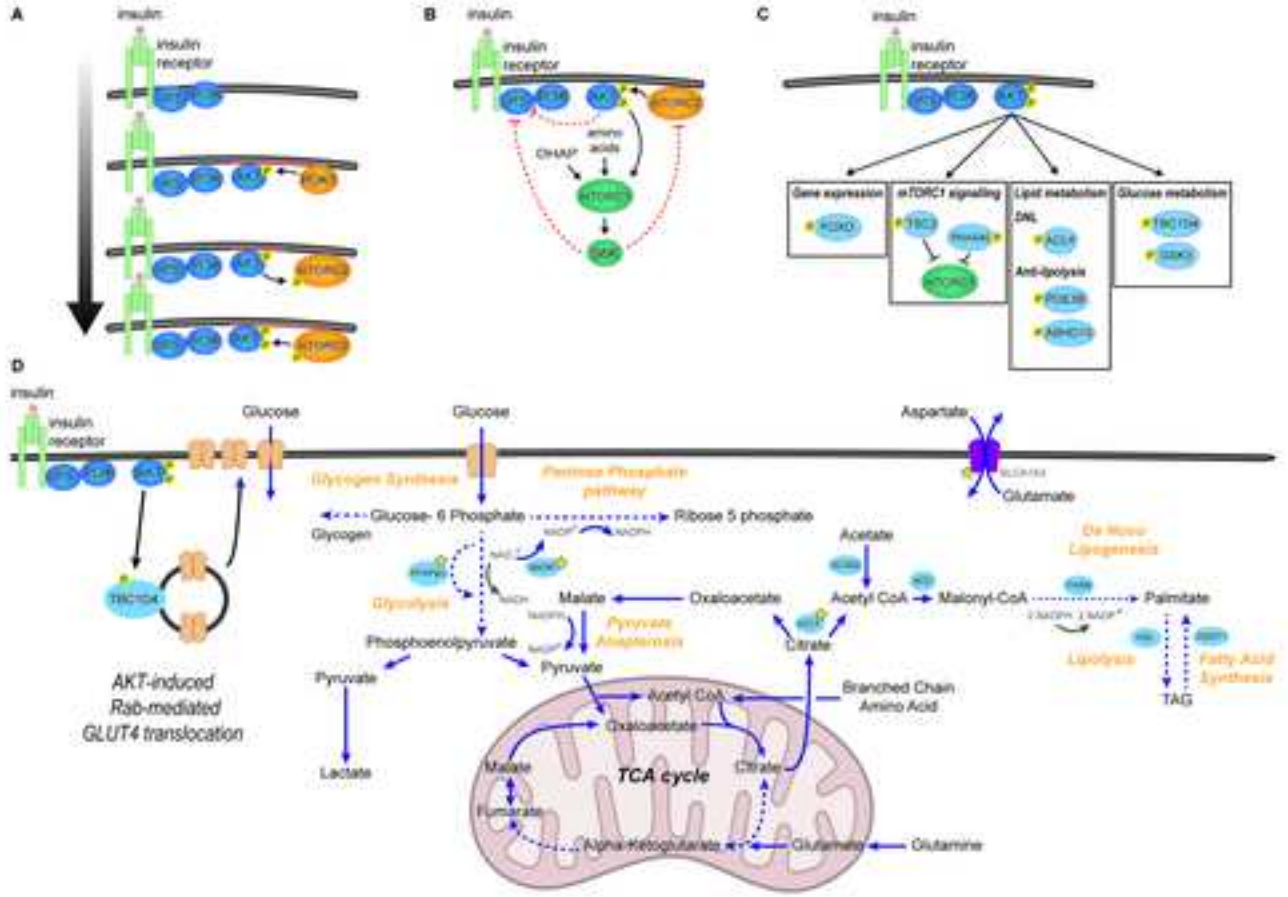
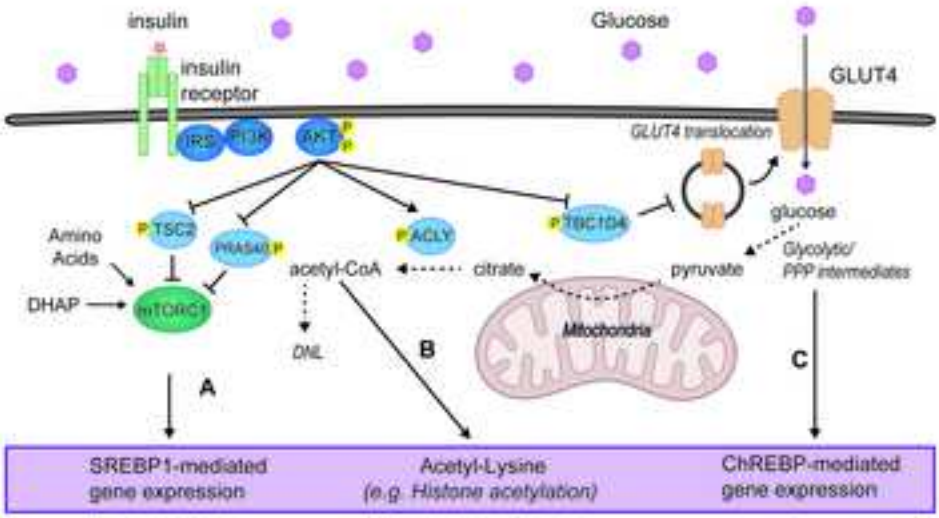


Figure 2



Outstanding Questions

- **How is insulin signaling and metabolism integrated in cellular time and space?** 'Omics analyses performed over time can unbiasedly reveal phosphorylation kinetics and metabolic fluxes, but do not provide details on subcellular localization. Understanding how signaling and metabolism are compartmentalized in cells and how signals are communicated across membranes is an important frontier.

- **How flexible are adipocytes in their use of carbon and nitrogen sources?** The substrates available for use in metabolic pathways may vary widely with diet, environment, or stress (e.g. hypoxia in obesity). As *in vivo* techniques advance it will be important to resolve how adipocyte metabolism works across a variety of diet, strain, and environmental conditions.

- **What is the role of adipose tissue heterogeneity and organ metabolite sharing?** Adipose tissues contain many different types of cells--even individual adipocytes within the same depot can vary metabolically. Moreover, adipose tissues work in concert with other organs. It will be important to understand how heterogenous cell populations within a single tissue work together, and how adipose tissues work with other organs to maintain metabolic homeostasis.

- **What causes adipose tissue insulin resistance?** Part of the challenge in answering this question is the extensive crosstalk between signaling, metabolism, and gene expression. For example, changes in metabolism can alter signaling responses and gene expression, raising the possibility that a primary defect in metabolism could drive insulin resistance. Disentangling this complex crosstalk is essential to understanding the molecular pathology of insulin resistance.

- **What are the critical signaling metabolites in adipocytes**? Numerous examples of metabolites functioning as signaling molecules exist but understanding the full spectrum protein-metabolite interactions and their roles in insulin signaling and metabolic disease is in its infancy.