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Review

Microtubules in insulin action: what's on the tube?

Skylar R. Batty[1] and Paul R. Langlais [2,*]

Microtubules (MT) have a role in the intracellular response to insulin stimulation and subsequent glucose transport by glucose transporter 4 (GLUT4), which resides in specialized storage vesicles that travel through the cell. Before GLUT4 is inserted into the plasma membrane for glucose transport, it undergoes complex trafficking through the cell via the integration of cytoskeletal networks. In this review, we highlight the importance of MT elements in insulin action in adipocytes through a summary of MT depolymerization studies, MT-based GLUT4 movement, molecular motor proteins involved in GLUT4 trafficking, as well as MT-related phenomena in response to insulin and links between insulin action and MT-associated proteins.

Insulin and microtubules: friends or frenemies?

Postprandial elevated blood glucose triggers the release of insulin from the pancreatic beta cell, resulting in an insulin-stimulated influx of glucose into muscle cells and adipocytes. Failure of insulin target tissues to respond to insulin ('insulin resistance') contributes to type 2 diabetes mellitus and a host of associated complications that, when left untreated, can be fatal (reviewed in [1–3]). Insulin binding to the cell surface insulin receptor (IR) leads to activation of phosphatidylinositol 3-kinase (PI3K). Subsequent activation of signal transduction cascades stimulates an increase in the number of **GLUT4** (see Glossary) molecules at the PM, resulting in an influx of glucose into muscle and adipose tissue (reviewed in [4]).

GLUT4 exiting the perinuclear region becomes packaged into a vesicle so highly specialized that its trafficking is still being elucidated after over 30 years of characterization [5] (Figure 1A). Once packaged, the GLUT4 vesicle is sorted through various compartments that contribute to the management of insulin-controlled glucose transport in target tissues, a broad field that has been reviewed at length elsewhere [3,5–8]. Intracellular insulin action incorporates multiple cytoskeletal structures, since inhibiting either **actin** or **MT** polymerization leads to decreased insulin-stimulated GLUT4 trafficking and glucose transport, a topic we explore in this review.

MTs are found throughout the cell as 25-nm hollow tubes comprising alpha- and beta-tubulin dimers that exhibit polarization, with the **minus-end** being anchored and the growing **plus-end** acting as the site of tubulin polymerization (Figure 1B). MT dynamics, such as assembly and disassembly rates as well as duration of stability, are controlled by **microtubule-associated proteins** (MAPs), a large family of proteins with several notable subgroups (reviewed in [9]). While many MAPs localize to the minus-end or along the length of the MTs (the lattice), the **MT plus-end tracking proteins** (+TIPs) are found at the growing plus-end tip of MTs. The +TIPs are a functionally diverse group of almost 20 proteins and, as such, their regulation and cooperativity have been explored in detail (reviewed in [10,11]).

Here, we narrow our focus to discuss research findings on the subject of MT involvement in adipocyte insulin action and GLUT4 trafficking, a topic we present in hopes of providing an answer to the question: 'what is the deal with MTs and insulin?'.

Highlights

The work on microtubules (MT) and insulin can be thought of as one huge mystery novel, impossible to understand by reading a page or perhaps just one chapter; only after finishing the entire saga does a clear picture emerge.

MT involvement in the acute insulin response that coordinates glucose transporter 4 (GLUT4) arrival at the plasma membrane (PM) is controversial, largely in part due to experiments with MT-depolymerizing agents.

This review addresses insulin-stimulated GLUT4 translocation and the literature on GLUT4 movement along the cytoskeleton.

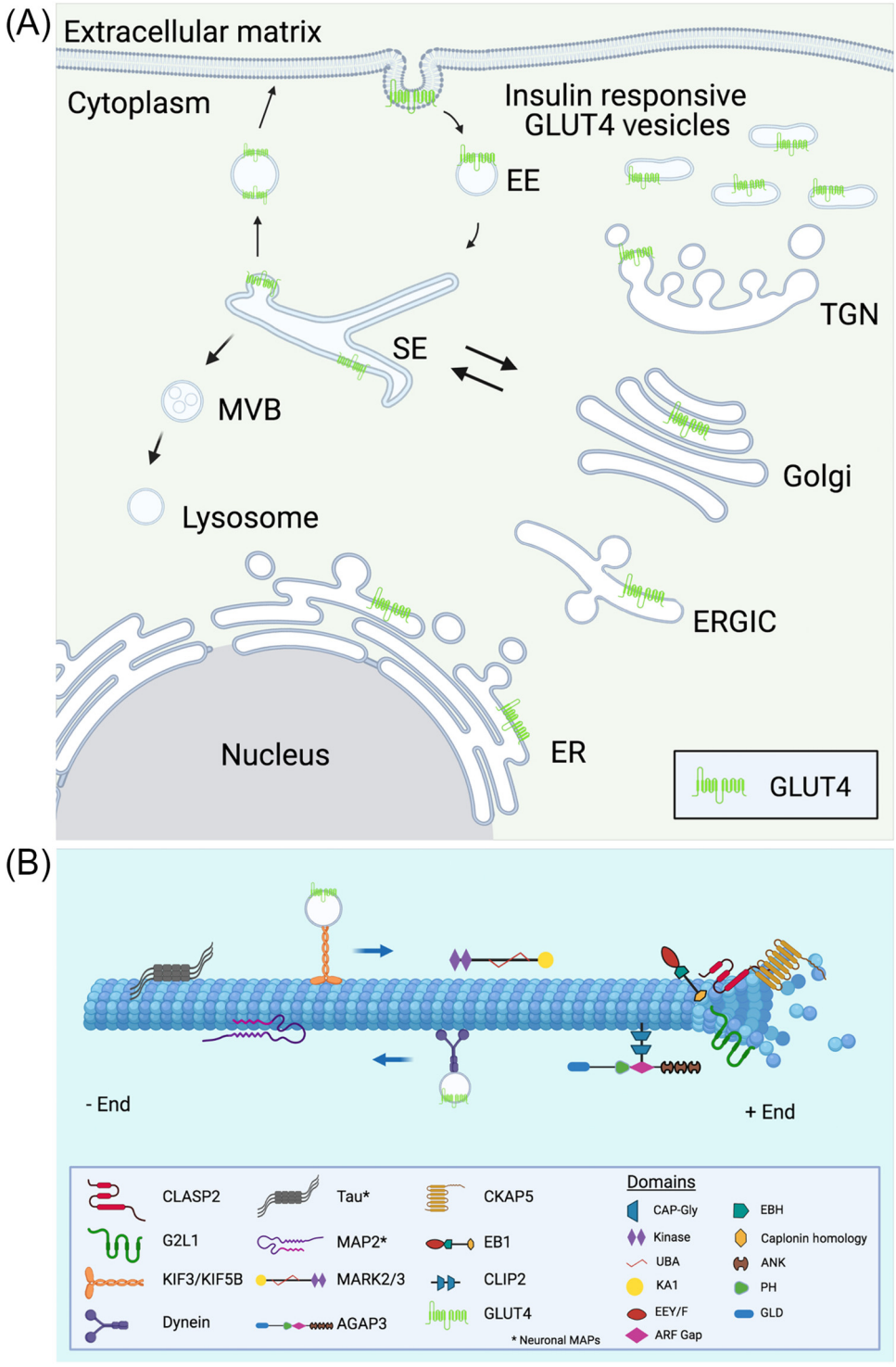
Recent findings have discovered an entire network of MT-associated proteins responsive to insulin.

In addition to an effect on MT-associated proteins, insulin also has effects on MT modifications and dynamics.

MT and actin converge at the PM to deliver GLUT4.

[1]Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ, USA
[2]Department of Medicine, Division of Endocrinology, University of Arizona College of Medicine, Tucson, AZ, USA

*Correspondence:
langlais@deptofmed.arizona.edu
(P.R. Langlais).



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Glossary

Actin: filamentous cytoskeletal element that can underlie the PM.

Colchicine, nocodazole, vinblastine: MT-depolymerizing agents used to study the function of MTs in a given biological context.

Cytolinker: protein capable of connecting multiple cytoskeletal elements.

Docking: association of the GLUT4 vesicle with the SNARE complex at the PM.

Dynein: processive motor protein that transports cellular cargo (including GLUT4 vesicles) toward the minus-end of MTs.

Fusion: fusion of the GLUT4 vesicle with the PM.

Glucose transporter 4 (GLUT4): responsible for insulin-stimulated glucose transport in muscle and adipose tissue.

Insulin-responsive GLUT4 pool: the pool of GLUT4 responsible for the mass efflux of GLUT4 to the PM in response to insulin; we have avoided the use of the acronyms 'GSV – GLUT4 storage vesicle', 'IRV – insulin responsive vesicle', 'GSC – GLUT4 storage compartment' and simply used 'GLUT4 vesicle' as the term and placed this phrase in context.

Kinesin: processive motor protein that transports cellular cargo (including GLUT4 vesicles) toward the plus-end of MTs.

Latruculin B (Lat-B): actin polymerization inhibitor useful for studying the involvement of the actin cytoskeleton in a biological process.

Microtubule (MT): hollow cytoskeletal tube that functions to provide both structure for the cell and tracks for the transport of cellular cargo.

Microtubule plus-end interacting proteins (+TIPs): proteins that track the growing plus end of the MT where tubulin polymerization occurs although not all +TIPs are alike, with some being tight to the extreme tip while others are further back.

Microtubule-associated proteins (MAPs): proteins that can decorate any part of the MT; used as an overall generalized term.

Minus-end: the base of the MT.

Myo1c/MyoVa: nonprocessive motor proteins that transport cellular cargo (including GLUT4 vesicles) along actin.

pHluorin: an excellent biochemical tool used to separate vesicle fusion from vesicle trafficking by taking advantage of

sensitivity to differences in pH between the lumen of a vesicle versus that of the extracellular media.
Plus-end: growing tip of the MT.
Spheroid: adipocytes grown in 3D culture.
TIRF zone: first 100–200 nM inside the PM.

Microtubule-disrupting agents in insulin action: disrupters indeed

MT-depolymerizing agents act in different manners, given that **colchicine** complexes with free tubulin and binds MT ends to stop MT polymerization; **vinblastine** causes tubulin aggregation; while **nocodazole** binds β-tubulin and prevents MT assembly/disassembly. MTs were first implicated in insulin action during the 1970s, after reports of an inhibitory effect of colchicine on insulin-stimulated glucose incorporation into glycogen and lipids in fat cells, although colchicine did not significantly affect insulin-stimulated glucose oxidation [12,13]. Another initial study found that colchicine and vinblastine both delay the activation of glucose transport in isolated rat adipose cells, and these effects were not caused by delayed insulin receptor coupling [14]. Almost 30 years later, initial time-lapse confocal microscopy studies in 3T3-L1 adipocytes observed that GLUT4 vesicles travel distances corresponding to those associated with MTs [15].

Effects on glucose transport

Measurement of the effects of MT-disrupting agents on insulin action has been done by assessing both glucose transport and GLUT4 translocation via several methods (Table 1). Numerous studies have documented decreased insulin-stimulated glucose transport because of MT depolymerization via radioactive glucose transport assays [15–18] and oil-flotation assays [19], in 3T3-L1 adipocytes and primary rat adipocytes. Additional agents that affect MTs, including BAPTA and dimethyl-BAPTA (reagents that chelate and sequester intracellular calcium), were also found to decrease glucose transport in response to insulin treatment in 3T3-L1 adipocytes [20]. Studies using high doses of nocodazole should be avoided because high doses of nocodazole inhibit glucose transport due, in part, to the nonspecificity of the drug on GLUT4 activity [21–23].

Experiments on GLUT4 at the PM

MT disruption has also been found to decrease the amount of GLUT4 at the PM in response to insulin, which has been quantified through fluorescence intensity measurements of GLUT4-eGFP in fixed [15] and live [16,18] cell settings, and with a Myc epitope in an exofacial loop of the transporter [24]. Other studies observed decreased insulin-stimulated GLUT4 translocation through PM lawn assays coupled with fluorescence intensity quantification of anti-GLUT4 antibodies [25], PM fractionation followed by western blot and densitometry quantification [26], tracking of GLUT4-YFP [27] or GFP-GLUT4 [15] translocation in live cells, GLUT4 fluorescence quantification at the PM through measurement of percent-positive cells at the PM [17], and the use of a carefully designed rating system of endogenous GLUT4 fluorescence in primary rat adipocytes [19]. Notable contradictions to reduced GLUT4 translocation in response to MT disruption were also reported early on [21–23], although subsequent publications diverged from these findings and instead consistently reproduced inhibitory effects of MT disruption on GLUT4 trafficking to the PM.

Effects on GLUT4 trafficking

Nocodazole treatment does not affect the basal insertion of GLUT4 into the PM, indicating that MT-based GLUT4 trafficking is not part of the slow, basal recycling of GLUT4 with the PM (or the implied basal internalization of GLUT4 [28]). In addition, GLUT4 shuttles between a specialized,

Figure 1. A glucose transporter 4 (GLUT4) and microtubule overview. (A) GLUT4 transits through the perinuclear compartment comprising the endoplasmic reticulum (ER), the endoplasmic reticulum–Golgi intermediate compartment (ERGIC), the Golgi, and the *trans*-Golgi network (TGN) to eventually become packaged as an insulin-responsive GLUT4 vesicle. GLUT4 also takes part in the endosomal recycling system via early endosomes (EE), which transfer through sorting endosomes (SE) to transit back to the TGN for repackaging. GLUT4 can also be transported through the multivesicular body (MVB) sorting system to the lysosome for degradation. The pathway depicted is centered on a current consensus model for GLUT4 trafficking [99]. (B) The microtubule is polarized, with the minus-end (-End) serving as the base, while the plus-end (+End) is the site of MT growth resulting from the addition of tubulin dimers. The different proteins depicted are MT-associated proteins (MAPs) that have a variety of individual molecular properties, although they have one thing in common in that they have all been linked to insulin action. Figure created using BioRender (https://biorender.com/).

Table 1. Reported effects of MT-depolymerizing agents on glucose transport and GLUT4 localization

Agent	Glucose transport		GLUT4 translocation		
	Decreased	No effect	Decreased	No effect	Basal GLUT4 redistribution from perinuclear region
Nocodazole	[16][a] [26][a] [19][b] [17][a] [18][a] [100][a] [23][c]	[21][a] [22][a]	[25][a] [19][b] [17][a] [18][a] [100][d] [28][a] [101][a] [102][e]	[103][a] [21][a] [22][a] [23][c]	[34][a] [21][a] [22][a] [23][a] [17][a] [29][a]
Colchicine	[15][a] [17][a] [100][d]	[23][c]	[27][a]	[22][a]	[15][a]
Vinblastine	[15][a]		[15][a]	[22][a] [103][a]	
BAPTA and dimethyl BAPTA	[20][a]				[20][a]
Cytochalasin B	[16][a]				
Latrunculin B			[25][a]		
Vm-1A					[34][a]

[a]3T3-L1 adipocytes.
[b]Primary rat adipocytes.
[c]Rat skeletal muscle.
[d]L6 myotubes.
[e]L6 myocytes.

insulin-responsive storage compartment and the endosomal system in the basal state, a distribution that is also unaffected by nocodazole [19,28]. MT depolymerization influences GLUT4 localization in the basal state by causing dispersion of GLUT4 from the perinuclear region [16,17,25]. Nocodazole interference with insulin-stimulated GLUT4 trafficking to the PM while simultaneously causing perinuclear GLUT4 dispersion suggests that the perinuclear localization of GLUT4 is integrated in insulin-regulated GLUT4 translocation [17], especially since GLUT4 perinuclear localization is decreased upon insulin stimulation [29]. This has been challenged in the literature [21], and by the fact that freshly isolated rat adipocytes have been observed to exhibit punctate GLUT4 localization throughout the cytoplasm near the PM, whereas 24-h incubation in cell culture changes GLUT4 localization to the perinuclear region [30,31]. This perinuclear GLUT4 topic had faded from view until only recently, when direct mechanistic evidence of perinuclear involvement in insulin-stimulated GLUT4 mobilization was reported. Through the clever use of the photoconvertible protein mEos3.2 [32] attached to GLUT4 in 3T3-L1 adipocytes, live cell studies revealed that AKT signals to Rab10 and TBC1D4 to mediate insulin-increased GLUT4 mobilization from the perinuclear region. The authors proposed that the initial insulin- stimulated surge in GLUT4 appearance at the PM may be due to readily available GLUT4 proximal to the PM, whereas a secondary wave of GLUT4 may source from the perinuclear region, either through GLUT4 vesicle biogenesis or perhaps accelerated GLUT4 vesicle movement along MTs. Additional insight was provided when experiments combining Rab10 knockdown with nocodazole treatment led to an additive effect on inhibiting insulin-stimulated GLUT4 appearance at the PM, suggesting that Rab10-mediated release of perinuclear GLUT4 vesicles and PM-directed transport of the perinuclear GLUT4 vesicle are independent of MTs. With the prevalence of data in the literature indicating that long-range transport of the GLUT4 vesicle occurs on MTs, the combinatorial Rab10 knockdown/nocodazole data support the findings that Rab10, in addition to a role at the perinuclear region, also regulates GLUT4 vesicle arrival at the PM in 3T3-L1 adipocytes [33].

Nocodazole-based relocalization of GLUT4

It is important to note that MT disruption with nocodazole [15–17,25,34] results in increased GLUT4 localization proximal to the actin cytoskeleton at the PM [22] and also immobilization for longer periods of time than normal of GLUT4 vesicles already localized at the PM [18]. This effect is not accompanied by an increase in glucose transport, indicating that this MT depolymerization-based relocalized GLUT4 is not inserted into the PM [17,22]. This nocodazole-induced GLUT4 relocalization near the PM may explain the finding that constitutively active AKT stimulates GLUT4 insertion into the PM in the absence of an intact MT network [29]. This phenomenon could result from the nocodazole-based relocation of GLUT4 proximal to, and immobilized at, the PM and activation of AKT-sensitive GLUT4 vesicles and PM molecular events controlling **fusion**. Paradoxically, this effect bypasses the insulin-regulated actin dynamics that are critical for effective GLUT4 localization at the PM [17,25], since AKT is reportedly not involved in the insulin-stimulated actin response [35]. Nocodazole-induced GLUT4 relocalization close to the PM may also explain why platelet-derived growth factor (PDGF), which cannot normally stimulate GLUT4 translocation, enhances GLUT4 translocation and glucose transport in nocodazole-treated 3T3-L1 adipocytes [17].

TIRFM analysis

The role of MTs in insulin action has also been studied with total internal reflective microscopy (TIRFM), which images just the first 100–200 nM of the cell interior proximal to the PM, known as the '**TIRF zone**' [36]. Nocodazole treatment of 3T3-L1 adipocytes has been observed with TIRFM to reduce insulin-stimulated intensity of GLUT4 at the PM to various levels [18,36], and a time-lag of GLUT4 appearance also occurs [36]. Using IRAP-**pHluorin** transfected cells, a construct that allows for the study of GLUT4 vesicle fusion with the PM [37], nocodazole treatment did not significantly decrease the number of fusion events stimulated by insulin, leading to the conclusion that MTs are not vital for the insertion of GLUT4 into the PM, but instead may have a more important role in site selection for delivery of GLUT4 before fusion [36].

Effects on insulin signal transduction

The involvement of the MT network in insulin signal transduction has also been investigated using MT-depolymerizing agents. Nocodazole treatment of 3T3-L1 adipocytes does not affect insulin-stimulated phosphorylation of the insulin receptor β-subunit, IRS-1, GLUT1 translocation to the PM, c-Cbl phosphorylation, or AKT phosphorylation at Ser473 [17,19,26]. However, insulin-stimulated phosphorylation of AKT at Thr308 and the kinase activity of AKT does decrease with nocodazole treatment, indicating that MTs are required for full activation of the kinase that controls AKT and PDK1, and that the effects of MT depolymerization on glucose transport and GLUT4 trafficking are mediated, in part, by interfering with signal transduction elements [29].

Interpreting the disrupters

With MT-depolymerizing agents causing simultaneous changes in GLUT4 localization, GLUT4 activity, and losses in insulin signal transduction, it is difficult to answer individual research questions using these reagents. Each of the changes that are induced contribute to a compounded effect on insulin action in adipocytes that is better interpreted as a sum of individual parts and not as independent, singular events. As such, it can be argued that conclusions about mechanistic events in insulin action using these agents must be made with caution.

GLUT4 trafficking along MTs

With the discovery of the insulin-sensitive glucose transporter GLUT4, understanding the role of the cytoskeleton in the trafficking of GLUT4 began, a pursuit that benefitted with each landmark technological development within the art of microscopy. Immunoelectron microscopy of GLUT4

vesicles from insulin-sensitive fractions, isolated by an equilibrium density gradient from 3T3-L1 adipocytes, identified an association between α-tubulin and GLUT4-containing membranes [34]. Shortly after, GFP-tagged GLUT4 in 3T3-L1 fibroblasts and adipocytes were observed to undergo extended linear movements that corresponded to vesicle translocation along the cytoskeleton [15], findings that were reproducible by others [16]. These linear movements were not affected by cytochalasin D treatment, a potent inhibitor of actin polymerization, whereas treatment of the cells with colchicine and vinblastine blocked GFP-GLUT4 movement [15]. At about the same time, it was reported that GLUT4 binds polymerized tubulin *in vitro* [26], suggesting that MTs tether or track GLUT4. It was the subsequent fluorescent tagging of tubulin that led to the first reported instance of GLUT4 movement along MTs, a landmark finding which solidified that long-range movement of GLUT4 occurs on MTs [27]. Initially, increased frequency (but not velocity) of long-range GLUT4 movement along MTs in response to insulin stimulation was observed in time-lapse images, with bi-directional movement of up to 20 μm toward both the periphery of the adipocyte and the perinuclear region [27]. These long-range insulin-stimulated movements were not affected by inhibition of the PI3K pathway, and neither was GLUT4 collection at the cell periphery, although GLUT4 insertion into the PM was mostly blocked [24]. A decade later, with the use of advancements in live cell TIRFM and two-color tagging of GLUT4, the approach of GLUT4 to the PM was shown to be sensitive to PI3K inhibition [38], a discrepancy with the previous finding that may be explained by the improved sensitivity of TIRFM over the earlier imaging techniques. In addition to these 3T3-L1 findings, GLUT4 has also been reported to colocalize with MTs in isolated rat adipocytes from epididymal adipose tissue, an association that is decreased by insulin [19].

Basally, GLUT4 vesicles can scan the region proximal to the PM before returning to the cytosol. Alternate rates of GLUT4 movement along MTs have been observed in the TIRF zone [15,18,30,39], including 0.6 μm/s, corresponding to conventional **kinesin** [27], and 2.0 μm/s, a velocity typical of fast kinesin or **dynein** motors [40,41]. GLUT4 vesicle movement near the PM is halted by insulin stimulation [29–31], a mechanism attributed to immobilization of GLUT4 vesicles near the PM at sites that likely correlate with MTs [30]. With the development of reagents that separate vesicle trafficking from vesicle/membrane fusion (using the pH-sensitive fluorescence protein pHluorin [37]), GLUT4 insertion sites into the PM were observed to spatially correlate with MTs [36]. Basal long-range movements of GLUT4 vesicles were observed in the pHluorin study, as previously reported [30,31], although the GLUT4 vesicles rarely moved long distances before fusion [36]. The same study reported that nocodazole modestly reduced the insulin-stimulated intensity of GLUT4 in the TIRF zone and a time-lag in the appearance of GLUT4 was introduced [36]. Importantly, nocodazole treatment did not significantly decrease the number of GLUT4 vesicle fusion events stimulated by insulin, evidence that MTs do not participate in GLUT4 vesicle fusion with the PM. Therefore, MTs manage basal transport of the GLUT4 vesicle proximal to the PM and facilitate the availability of GLUT4 for sites at the PM selective for GLUT4 fusion [36], a notion supported by the finding that MT depolymerization blocks mobility of GLUT4 vesicles in the TIRF zone at the PM [18].

Cortical actin remodeling occurs in response to insulin stimulation in adipocytes, and disruption of this phenomenon inhibits glucose transport [42,43]. Inhibiting both actin and MTs concurrently completely blocks insulin-stimulated glucose transport, indicating that both cytoskeletal elements are integrated in GLUT4 trafficking [16,25]. Treatment of cells with the actin polymerization inhibitor **latrunculin B** (Lat-B) does not affect the magnitude or kinetics of insulin-stimulated GLUT4 translocation to the PM, suggesting that normal insulin-regulated delivery of GLUT4 vesicles close to the PM is independent of actin. By contrast, Lat-B treatment reduces GLUT4 exocytosis, establishing again that actin is vital for the exocytotic fusion of GLUT4 into the PM [43].

MT response to insulin

The data implicating MTs in insulin action discussed thus far have primarily focused on the role of MTs as a transport system for GLUT4-containing vesicles. However, studies have noted a direct response of MTs to insulin stimulation. Some of the first reported experiments found that α-tubulin is phosphorylated in response to insulin stimulation by the purified insulin receptor kinase [44]. Two distinct subpopulations of phosphorylated tubulin exist that exhibit different polymerization capabilities due to different tyrosine phosphorylation locations on the α-subunit [45]. A later study revealed that insulin stimulation promotes the polymerization of MTs in 3T3-L1 adipocytes independently of the PI3K and MAPK/ERK pathways, an event that relies on an intact actin cytoskeletal network. This polymerization of MTs is not reliant on the creation of new MTs through MT nucleation, but rather on the growth of established MTs [46]. The same study further elucidated the role of insulin receptor kinase using the tyrosine kinase inhibitor genistein, which significantly decreased MT polymerization in response to insulin, leading to the conclusion that MT polymerization is dependent on tyrosine kinase activity [46]. Further studies using TIRFM quantitatively identified both an increase in MT density and curvature within 200 nM from the PM in response to insulin stimulation, an effect that was independent of the actin network [36]. Conversely, using the MT plus-end binding protein CLASP2 as a marker, insulin treatment was shown to reduce the average velocity of the growing plus-end of MTs, implicating that, while insulin may increase gross MT polymerization and density, MTs polymerize slower in the presence of insulin [47]. MTs are dynamic, and transient periods of growth and shrinkage typify short-lived MTs [11], whereas other MTs can be stabilized and remain in place for longer. These longer-lived MTs can act to transport cellular cargo, and often have α-tubulin acetylation at lysine 40, a post-translational modification that protects MTs from mechanical stress [48,49]. With the amount of GLUT4 appearing at the PM during insulin stimulation, it did not come as much of a surprise when it was discovered that insulin stimulates α-tubulin acetylation at lysine 40 as well as MT stabilization in 3T3-L1 adipocytes, a mechanism regulated through a balance between GSK3 and rapamycin-sensitive mTOR [47]. This insulin-stimulated α-tubulin acetylation at lysine 40 peaks subsequent to steady-state GLUT4 appearance at the PM, perhaps as a result of the longer lived MTs needing mechanical repair after acting as a transport line for the GLUT4 vesicle for the duration of the insulin signal. As previously mentioned, α-tubulin has also been documented to associate with GLUT4 upon insulin stimulation [34], and GLUT4 has also been documented to bind polymerized tubulin *in vitro* [26]. Additionally, insulin treatment has been found to reduce the affinity of proteins for MTs, including the insulin signaling protein IRS1 [26].

Taken together, there are multiple direct effects of insulin stimulation on MTs, such as α-tubulin phosphorylation, MT polymerization, increases in MT density and curvature, MT reorganization, and α-tubulin acetylation and subsequent MT stabilization. Further research regarding the role of MTs in insulin action would help characterize the purpose behind these documented responses that, to date, have no defined function.

Motor proteins in insulin action

One of the earliest studies implicating a molecular motor on MTs involved in insulin action found that inhibition of the motor activity of the protein dynein, a motor that moves cellular cargo toward the minus-end of MTs, by slight acidification of the cytoplasm, resulted in dispersion of GLUT4 from the perinuclear region and reduced GLUT4 translocation to the PM in response to insulin [34]. It was later shown that insulin inhibits Rab5 activity and dynein binding to MTs in a PI3K-dependent manner, which was proposed to contribute to the inhibition of GLUT4 endocytosis and internalization in response to insulin in 3T3-L1 adipocytes [50].

Kinesins are processive, single-motor molecule proteins that transport cellular cargo over long distances toward the plus-end of MTs before detaching from the MT. Htau40 overexpression in 3T3-L1 adipocytes inhibits kinesin movement along MTs and reduces insulin-stimulated GLUT4 translocation by 65% in the first 3 min of the insulin response, a GLUT4 translocation time lag that is eventually overcome after 12 min of insulin stimulation [25]. Microinjection of neutralizing kinesin family member 3A (KIF3A) antibodies or KAP3A small interfering (si)RNA (a subunit of KIF3A) reduced insulin-stimulated GLUT4 translocation by 65% and 69%, respectively [51]. Insulin stimulation increases the interaction of Rab4 with KIF3A, and increases KIF3A interaction with MTs, a system mediated by PI3K and PKC-λ proposed to control KIF3A [51]. An additional ternary complex containing axin, TNKS2, and KIF3A has also been proposed to regulate KIF3A function in insulin action [52]. Kinesin family member 5B (KIF5B) has been reported to partially colocalize with GLUT4 vesicles in 3T3-L1 adipocytes, and dominant-negative mutants of KIF5B interfere with GLUT4 appearance at the PM [27].

Further studies in 3T3-L1 adipocytes investigated the role of TUG proteins in KIF5B-based transport of GLUT4 in response to insulin, in which the Usp25m protease was found to cleave TUG to form TUGUL, resulting in KIF5B modification by TUGUL, a process important for loading GLUT4 vesicles onto the MT motor KIF5B [53]. Another recent study identified a role for KIF5B *in vivo*, in which adipose-specific deletion of KIF5B in mice exacerbated insulin resistance resulting from diet-induced obesity [54]. Kinesin-based GLUT4 trafficking on MTs may be site specific, since GLUT4 proximal to the PM is halted by insulin, whereas GLUT4 from the perinuclear region is mobilized, although whether this difference is related to the multiple KIFs that are tied to insulin action has yet to be explored.

In addition to MT motor proteins, actin-based motor proteins have also been linked to GLUT4. An early study of **myosin 1c (**Myo1c) in insulin action found Myo1c in purified GLUT4 vesicles in 3T3-L1 adipocytes, and that the Myo1c–GLUT4 association is increased upon insulin stimulation. Myo1c cargo domain mutants and Myo1c-targeted siRNA were reported to inhibit GLUT4 translocation and glucose transport, respectively [24,55]. These findings were further confirmed by another study that used siRNA targeted toward Myo1c, in which a 65% reduction in GLUT4 translocation in response to insulin was documented [17]. Other studies identified a role for **myosin-Va** (MyoVa) in insulin-stimulated GLUT4 translocation, such that inhibition of MyoVa also results in decreased GLUT4 translocation to the PM [56,57].

These motor protein studies indicate that GLUT4 vesicles switch tracks from MTs to actin at some point during GLUT4 trafficking at the PM, a concept introduced before its time in 2001 [16,25]. Investigating the dynamics of Myo1c and kinesin motor proteins found that the intrinsic properties of Myo1c and kinesin-1 allow Myo1c to effectively halt kinesin-1 in areas of actin filament and MT intersections. Since Myo1c is slow and nonprocessive, such that Myo1c leaves the MT after each step, it is not likely that Myo1c actively transports the GLUT4 vesicle long distances during insulin action [58]. As an alternative to a function for GLUT4 vesicle transport, Myo1c causes extensive cortical membrane ruffling, an event mediated by actin reorganization [24]. Cortical actin acts as a barrier to vesicles, although actin reorganization relaxes the surface tension of this barrier to invite vesicle fusion. This explains why so much actin reorganization is visualized upon insulin stimulation and also why Myo1c proximal to the membrane surface triggers actin reorganization to promote GLUT4 vesicle fusion with the PM [59].

Microtubule-associated proteins in insulin action

Early MAPs linked to insulin action were MAP2 and tau, both of which are highly expressed in neurons. *In vitro* tyrosine phosphorylation of MAP2 and tau by the purified insulin receptor kinase

was shown to be increased by five-to-tenfold in the presence of insulin [44]. These findings were followed by a different study using 3T3-L1 adipocyte lysates with exogenous MAP2 purified from bovine brain, in which insulin stimulation was found to increase the serine phosphorylation of MAP2 [60]. This led the authors to name the kinase that phosphorylated MAP2 on serines 'microtubule-associated protein kinase' (MAPK) [61,62], a name that eventually transformed to 'mitogen-activated protein kinase' [63] and also 'extracellular signal-regulated kinase' (ERK) [64]. This *in vitro* MAPK/ERK-based phosphorylation of the MAPs (MAP2 and MAP4 specifically) was later shown to reduce the ability of these MAPs to induce *in vitro* tubulin polymerization into MTs [65], a finding that has yet to be explored in the context of insulin action. MAPK/ERK, a kinase with activity controlled by insulin, has also been demonstrated to associate with MTs [66–71], although whether a connection between MAPK/ERK, MTs, and insulin exists has not yet been established.

After the initial surge of investigation into MAPs and their associated kinases, and then the work on the molecular motor proteins, further research on a connection between MAPs and insulin action was largely delayed until 2010. At this time, a study investigating the protein PHLDB1 (also referred to as LL5α), reported that PHLDB1 translocates to the PM in response to insulin, and knockdown of PHLDB1 via siRNA decreased insulin-stimulated GLUT4 translocation and glucose transport in 3T3-L1 adipocytes, possibly through regulation of AKT [72]. While PHLDB1 itself is not a MAP, it has been documented to associate with the +TIP CLASP2 [73]. The possibility that CLASP2 has a role in insulin action was later brought into focus by a study that used mass spectrometry to perform an unbiased quantitative proteomics screen designed to detect insulin-responsive proteins in L6 myotubes via changes in protein phosphorylation in the presence of insulin. This study identified a 4.6-fold increase of CLASP2 in insulin-stimulated, phospho-antibody immunoprecipitates, the highest fold change of all the proteins screened in this study [74]. The same study also reported that siRNA-mediated knockdown of CLASP2 in L6-myotubes decreased insulin-stimulated GLUT4 presence at the PM, and that siRNA-mediated knockdown of CLASP2 in 3T3-L1 adipocytes inhibited insulin-stimulated glucose transport. These findings introduced a role for CLASP2 and +TIPs in insulin action, possibly through GLUT4 trafficking along MTs [74]. This study also reported that CLASP2 colocalizes with GLUT4 in areas of insulin-stimulated actin reorganization at the PM [74], which aligns with the model that the actin and MT cytoskeletal networks converge at areas of insulin-stimulated GLUT4 translocation to the PM. These findings led to the hypothesis that basal, dynamic MTs may be guided to specific CLASP2-targeted landing zones on the cell cortex upon insulin stimulation, situating GLUT4-containing vesicles proximal to the PM.

Additional investigations into CLASP2 were performed through interactome studies in 3T3-L1 adipocytes, which revealed that CLASP2 coimmunoprecipitates the MT-associated kinase MARK2, the MT-actin crosslinking protein G2L1, and the newly discovered MAP, SOGA1, among others [75]. Most of these CLASP2-related proteins were subsequently discovered to undergo insulin-induced changes in phosphorylation in 3T3-L1 adipocytes, including G2L1, MARK2, CLIP2, AGAP3, CKAP5, and EB1, findings that established a new network of insulin-affected MAPs, albeit with no known function in insulin action to-date [47]. CLASP2 and G2L1 colocalize at MT plus-ends in the basal state in 3T3-L1 adipocytes, whereas insulin stimulation induces CLASP2 and G2L1 MT trailing, a phenotype characterized by the +TIPs leaving their exclusive localization at the MT plus-end to instead trail along the MT lattice behind the plus-end [47]. Another study investigating CLASP2 in neurons also reported that insulin treatment increases the length of the CLASP2 comet (a phenomenon equivalent to the 'trailing' phenotype in 3T3-L1 adipocytes), and that these CLASP2-containing comet dashes along the MT lattice prominently localize to cortical areas near cell edges [76]. This cell periphery-based shift of

Box 1. Not all adipocyte models are created equal

A key factor for the 3T3-L1 model is full differentiation of fibroblasts into adipocytes, because fibroblasts do not endogenously express GLUT4 (Figure I). 3T3-L1 fibroblasts have been reported to both lack [84–87] and have [88–92] insulin responsiveness. However, the insulin response observed in fibroblasts is less than that of fully differentiated adipocytes.

Fibroblasts have increased recycling of exogenous GLUT4 to the PM in the basal state compared with adipocytes, whereas adipocytes have increased GLUT4 at the PM in response to insulin stimulation [88,92,93]. The rate of GLUT4 cycling in response to insulin in adipocytes is attributed to distinct pools of GLUT4 that are specified during the differentiation process [88], and this specification precedes the endogenous expression of GLUT4 [94]. Around 70–90% of GLUT4 in adipocytes is in the insulin-responsive pool, and 10–30% is in the actively recycling pool [93], whereas fibroblasts have upwards of 50% of their exogenous GLUT4 in the recycling pool [88]. Sortillin and TBC1D4/AS160 are involved in the formation of distinct GLUT4 pools during differentiation. Although sortilin is not expressed in fibroblasts, its expression is increased during differentiation, and correlates temporally with GLUT4 active-recycling becoming more restricted, representing the formation of the insulin-responsive pool [95]. Sortillin expression protects GLUT4 from lysosomal degradation and, in turn, results in more stable GLUT4, which is seen in adipocytes [93]. TBC1D4/AS160 has also been implicated in the formation of distinct GLUT4 pools during differentiation, because TBC1D4/AS160 expression is also induced during adipocyte differentiation and participates in releasing GLUT4 from the static pool upon insulin stimulation [95].

Differentiated 3T3-L1 adipocytes have heterogeneous multilocular lipid droplets, while isolated primary adipocytes are dominated by a unilocular lipid droplet and have a small cytoplasm between the lipid droplet and the PM [30]. GLUT4 in primary adipocytes differs from 3T3-L1 adipocytes, such that GLUT4 is dispersed throughout the cytoplasm instead of the perinuclear localization seen in 3T3-L1 adipocytes [30,96]. Freshly isolated primary adipocyte cytoplasmic distribution of GLUT4 becomes more perinuclear with increasing time in culture [30].

3D **spheroid** cultures allow for recapitulation of *in vivo* tissue morphology and function that 2D models cannot provide. A variety of 3D spheroid adipocyte culture approaches are available, including 3T3-L1, human adipose-derived stem-cells, and stromal vascular fractions. 3T3-L1 adipocyte spheroids have larger lipid droplets and increased expression of critical genes for adipogenesis compared with the other approaches [97]. Additionally, these cultures have downregulation of various extracellular matrix genes, a process that does not occur in 2D cultures, resulting in a more physiologically relevant model [98].

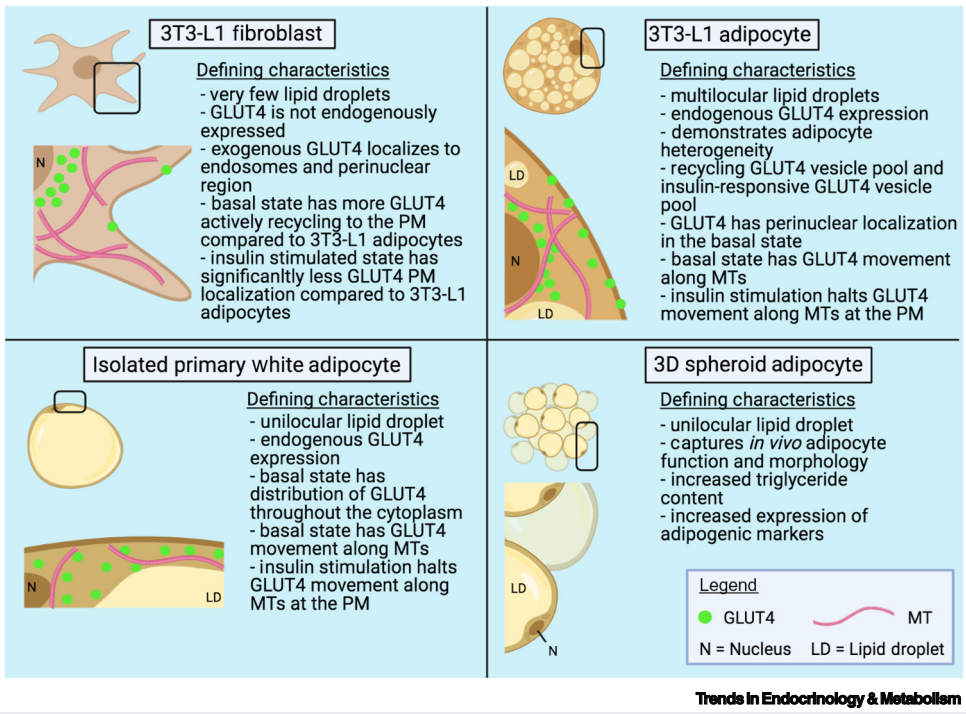

Figure I. Adipose models have bad customer service. There has been excellent work on breaking down the subtle nuances in glucose transporter 4 (GLUT4) trafficking between the different models available for researching adipocyte insulin action [93]. These comparative studies have led to keen insight into the different routes GLUT4 takes and have placed emphasis on understanding that regardless of the model chosen, attention must be paid to the fact that the cellular status will dictate the GLUT4 itinerary. For example, if the 3T3-L1 adipocytes are not fully differentiated, development of the GLUT4 trafficking system will stall out and result in experimental results that reflect some sort of confused intermediate GLUT4 trafficking system. Figure created using BioRender (https://biorender.com/).

CLASP2 from the MT plus-end to the lattice has also been observed in live migrating Ptk1 epithelial cells, in which lamella and lamellipodium-based CLASP2 lacked +TIP behavior and instead exhibited trailing via binding along the length of the growing MT lattice [77], reminiscent of

Key figure

Role of microtubules (MTs) in insulin action

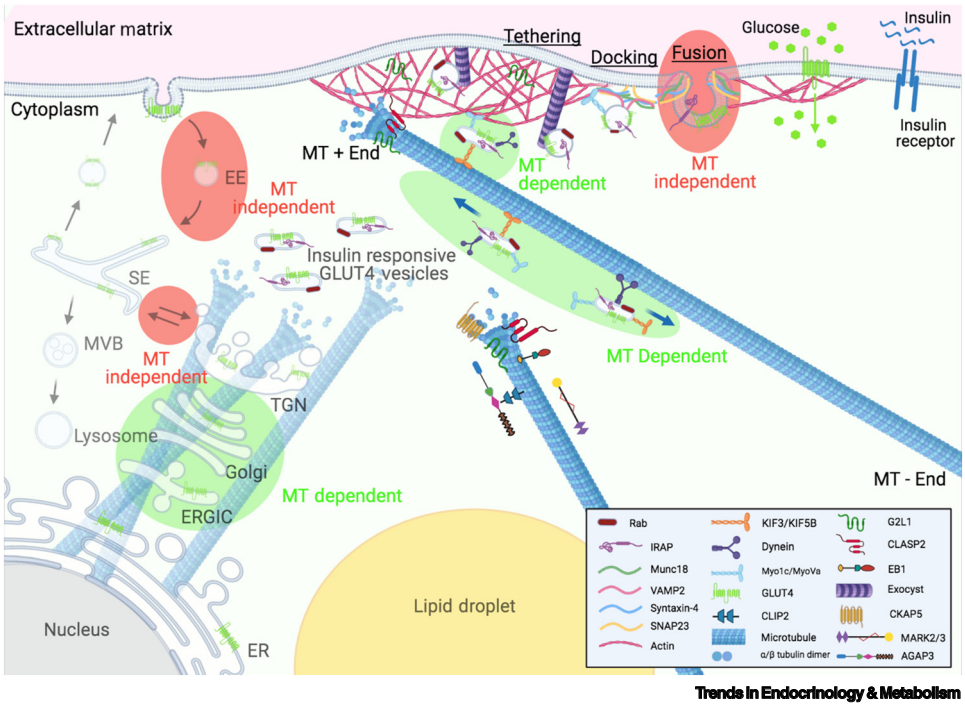

Figure 2. The model depicts that, upon completion of sorting, the insulin-responsive glucose transporter 4 (GLUT4) vesicle can ride the MT, scanning the inner surface of the plasma membrane (PM), readily available for quick action. This MT-based GLUT4 is separate from GLUT4 docked or tethered at the PM, and is also distinct from the MT-independent endosomal recycling GLUT4 pool. Upon insulin stimulation, MT-based translocation of the GLUT4 vesicle at the PM is arrested. At this point, a transfer from MTs to actin occurs, perhaps in part through a dominance of actin-based motors over MT-based motors, although little if any continued translocation occurs on actin and, instead, the **docking**/fusion machinery takes over and GLUT4 reaches the PM. Figure created using BioRender (https://biorender.com/).

the effect of insulin observed in neurons and 3T3-L1 adipocytes. Additionally, insulin treatment of neurons was also shown to induce CLASP2 binding to MT tips and increase the CLASP2 comet density [76].

It has been proposed that actin and MT dynamics coordinate at the PM to facilitate GLUT4 trafficking during insulin action [33,43,56,57,78–81]. The protein G2L1 is a fit in this proposed model of GLUT4 trafficking since G2L1 is known to regulate actin and MT crosstalk [82] and colocalizes with areas of actin and MT overlap [83]. Furthermore, as previously noted, G2L1 undergoes insulin-stimulated phosphorylation in 3T3-L1 adipocytes. G2L1 colocalizes with the insulin-responsive +TIP CLASP2 on the plus-end of the MT in 3T3-L1 adipocytes and also separately from MTs at areas of insulin-stimulated actin reorganization at the PM [47]. The limited evidence produced thus far regarding MAPs, +TIPs, and cytoskeletal crosslinking proteins designates G2L1 as a prime candidate for further investigation for its potential role in insulin action, specifically because it may serve as a **cytolinker** between actin and MTs, each of which are integral to GLUT4 translocation.

Concluding remarks and future perspectives

Our intention in writing this review was to summarize findings on the case of insulin versus MTs. Countless individual, specific experiments have directly addressed the relationship between MTs and insulin in the 30+ years of literature on the topic. To summarize, we present yet another model of GLUT4 translocation, one that incorporates elements of a current scheme in the field [99], and highlights consensus research findings about MTs (Figure 2, Key figure).

The next questions are simply where, how, and why (see Outstanding questions)? Where is the signaling in all of this dynamic MT action: the proteins, TBC1D4/AS160, actinin-4, or TUG? How do the MTs 'know' where to go? Why is insulin affecting the phosphorylation of all these MAPs and what are all these effects of insulin on MTs? What are they for? Does any of this happen *in vivo*, or is all of this just some artifact of a far-from-reality *in vitro* cell system (Box 1), with a cytoskeleton that lacks real-world intervention from the extracellular matrix? Now that we have discovered this new insulin-responsive MAP network, are these proteins dysfunctional or abnormally responsive in insulin resistance?

So, back to the original question 'what is the deal with MTs and insulin?'. A lot, apparently.

Declaration of interests

None declared by authors.

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Outstanding questions

How do actin and MTs intersect to provide a direct cytoskeletal path for GLUT4 to reach the PM in response to insulin? Are cytolinker proteins, such as G2L1, involved? Can we visually capture the phenomenon of GLUT4 vesicles switching cytoskeletal tracks at the PM in response to insulin in live cells, and tease apart the proteins that coordinate this event?

What is the implication of the multiple kinesin and myosin motor proteins regulating GLUT4?

How is the MT network involved, or not, in the various insulin signaling pathways? How is signaling integrated into the capture of GLUT4 along MTs at the PM?

Why does insulin cause an increase in MT density and stabilization, while simultaneously causing a decrease in MT velocity? In addition, what protein pathways are responsible for these effects? Are they involved in glucose transport and storage of lipids as triglycerides, including inhibition of lipolysis, or are they solely mitogenic, do they happen *in vivo*, and are they altered in insulin resistance?

What is the purpose of +TIP trailing in response to insulin? Further investigations will be necessary to underpin the biological significance of this phenomenon as it pertains to insulin action.

Are 3T3-L1 adipocyte models reliable to test GLUT4 translocation in response to insulin since these models lack several *in vivo* features and have a different basal GLUT4 localization (perinuclear) compared with primary adipocytes (even cytoplasmic distribution)? Do 3D spheroid adipocytes allow for integration of extracellular matrix signals to the cytoskeleton that are lost in 2D cultures?

What are the differences and similarities in MT regulation between the two insulin target tissues of muscle and adipose tissue?

What is happening to MAPs in response to insulin *in vivo* in humans and is their function altered in insulin resistance?

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