Trends in Endocrinology & Metabolism

CellPress

Review

Update on GLUT4 Vesicle Traffic: A Cornerstone of Insulin Action

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Glucose transport is rate limiting for dietary glucose utilization by muscle and fat. The glucose transporter GLUT4 is dynamically sorted and retained intracellularly and redistributes to the plasma membrane (PM) by insulin-regulated vesicular traffic, or 'GLUT4 translocation'. Here we emphasize recent findings in GLUT4 translocation research. The application of total internal reflection fluorescence microscopy (TIRFM) has increased our understanding of insulin-regulated events beneath the PM, such as vesicle tethering and membrane fusion. We describe recent findings on Akt-targeted Rab GTPase-activating proteins (GAPs) (TBC1D1, TBC1D4, TBC1D13) and downstream Rab GTPases (Rab8a, Rab10, Rab13, Rab14, and their effectors) along with the input of Rac1 and actin filaments, molecular motors [myosinVa (MyoVa), myosin1c (Myo1c), myosinIIA (MyoIIA)], and membrane fusion regulators (syntaxin4, munc18c, Doc2b). Collectively these findings reveal novel events in insulin-regulated GLUT4 traffic.

Trends

Insulin promotes GLUT4 redistribution from recycling endosomes and specialized intracellular compartments to the plasma membrane (PM).

Signaling bifurcates downstream of phosphatidylinositol 3-kinase towards Akt and Rac. Akt signaling leads to activation of Rab10 in adipocytes and Rab8a and Rab13 in myoblasts. Rab10 acts at several steps in GLUT4 translocation, whereas Rab8a promotes GLUT4 exit from the perinuclear region and Rab13 promotes tethering of GLUT4 vesicles near the PM.

Rac1 signaling induces cortical actin remodeling that tethers GLUT4 vesicles beneath the PM. Additional proteins may also fine-tune actin remodeling through tropomyosin3.1 and tropomodulin3 via Akt2 input.

Insulin increases [Ca^{2+}] beneath the PM, which may regulate the SNARE complex that mediates the final step of GLUT4 vesicle fusion with the PM.

GLUT4: The Predominant Insulin-Responsive Glucose Transporter

Insulin stimulation of glucose uptake in muscle and fat tissues is one of the paramount actions of the hormone, essential for energy storage. Some 30 years ago, it was discovered that the increase in glucose uptake is brought about by a change in the distribution of glucose transporters from intracellular storage vesicles to the PM of rat adipocytes [1,2] skeletal muscles [3,4], and fat and muscle cell lines [5,6]. Following the cloning of GLUT4 (SLC2A4), the major glucose transporter in muscle and fat, it was confirmed that vesicular traffic mediates its redistribution in response to insulin, a process generically called 'GLUT4 translocation'. In skeletal muscle, exercise/muscle contraction also causes redistribution of intracellular GLUT4 to the PM [7], although in this case glucose is used for energy supply.

Glucose transport is rate limiting for postprandial glucose utilization by fat and muscle tissues and GLUT4 translocation is deficient in type 2 diabetes. Despite worldwide efforts to elucidate the mechanism of GLUT4 cycling and signals regulating it, no current antidiabetic treatments directly target muscle/fat glucose transport. Therefore, it is important to further clarify the full extent of the mechanisms regulating GLUT4 translocation to identify potential targets for antidiabetic therapy. Here we build on previous reviews [8–11] to provide an update on the major findings of the past 5 years that have advanced our knowledge of three aspects: the nature of GLUT4 intracellular compartments; insulin-derived signals regulating GLUT4 cycling; and specific events at the cell surface.

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Intracellular Compartments Harboring GLUT4

Where Is GLUT4 Localized Inside Muscle and Fat Cells?

Immunofluorescence studies reveal that, in the basal state (in the absence of any stimuli), GLUT4 locates to a perinuclear depot and cytosol-dispersed vesicles within adipose and muscle cells, with only a small proportion at the PM. Early electron microscopy visualization revealed GLUT4 in intracellular vesicles, cisternae, tubules, and sacs. In response to insulin, about 20% (in muscle) or 50% (in fat) of the intracellular GLUT4 rapidly reaches the PM. Biochemically about 90% of the intracellular GLUT4 separates equally into two intracellular membrane fractions, one containing elements of recycling endosomes (RE) and denoted by the transferrin receptor, the other including some markers more typical of the trans-Golgi network (TGN) and the endoplasmic reticulum–Golgi intermediate compartment, depleted of transferrin receptor and enriched in the vesicular SNARE (v-SNARE) protein VAMP2 and insulin-responsive amino peptidase (IRAP), in addition to a few other proteins, described below. This has led to the concept that GLUT4 largely populates a general recycling compartment as well as a specialized or 'storage' compartment. With insulin stimulation this storage compartment is more markedly depleted as GLUT4 levels increase at the PM. This compartment has been termed GLUT4 storage vesicles (GSV) and/or insulin-responsive vesicles (IRV); however, some studies suggest that the storage compartment (GSV) feeds into IRV, which are the vesicles that reach the PM in response to insulin. The latter nomenclature is adopted in this review and assumes that the perinuclear compartment contains the GSV and the cytoplasmic and more peripheral vesicles represent IRV.

Despite the longstanding and wide interest in GLUT4 traffic, the biochemical identity of the storage compartment is still being refined. The search for specific markers of GSV and IRV has been compromised by difficulty in isolating each fraction, although recently developed fractionation protocols may achieve this goal [12]. Subcellular fractionation analysis has suggested that cellugyrin populates the sorting endosomes (SE) that generate GSV, which are instead enriched in sortilin [13]. The proteome of intracellular membranes highly enriched in GLUT4 reveals as major integral proteins IRAP, low-density lipoprotein receptor-related protein 1, and VAMP2 [14]. VAMP2 may be the best determining marker of the insulin-regulated pool, as in addition to segregating with the membrane fraction that is depleted in insulin-stimulated cells it is also the fusogen involved in insulin-dependent insertion of GLUT4 into the membrane, but not in the maintenance of the steady-state level of surface GLUT4 that obeys constitutive recycling [15]. The protein IRAP correlates with GLUT4 in all of its intracellular compartments and hence is not an exclusive GSV or IRV marker, but is a useful GLUT4 surrogate for the study of vesicle exocytosis [13]. These GLUT4-enriched vesicles also contain sortilin and syntaxin6, both of which have been connected with diverse aspects of GLUT4 traffic. In this context, it was recently demonstrated that sortilin mediates retrograde transport of GLUT4 to the TGN as well as its retrieval from lysosomes, through interactions with retromer [120]. Additional components include VAMP3, SCAMPs, B2M, SERPINA1, COPA, GCDN/MTPN, AIFM2, and tumor suppressor candidate 5 (TUSC5), which await functional assignment or may participate more generally in overall intracellular traffic. In this context two recent studies propose that TUSC5 contributes to GLUT4 vesicle fusion, based on gain- and loss-of-function studies [16,17] and future work should define whether TUSC5 specifically regulates GLUT4 traffic or has a more generalized input into recycling.

Are There Static and Dynamic Pools of GLUT4?

Extensive kinetic studies reveal that, in the basal state, any GLUT4 at the PM rapidly internalizes to reach first the general recycling compartment and then the storage compartment. However, it is still debated whether GLUT4 in this compartment is static or whether it recycles slowly back through the RE to the PM. The static and dynamic models of GLUT4 traffic were reviewed earlier [18] and continue to be the subject of further investigation, with the proportion of recycling varying from 20% to near 100% depending on cell type and culture conditions.

The Dynamic Retention Model. The extent of GLUT4 recycling to the PM in the absence of stimuli is more complete in muscle cells than in adipocytes. However, in both cell types kinetic and biochemical scrutiny supports the concept that insulin regulates at least two intracellular events: an increase in available IRV and their mobilization to the PM. Dynamic studies of GLUT4 cycling back from the PM show that the transporter enters the RE to then reach the GSV aided by syntaxin6 [19]. Hence, GSV maintenance is an ongoing and dynamic process. With insulin stimulation the perinuclear compartment spreads out and cytosolic vesicles disperse further, with 15% or 40% of the total GLUT4 fusing with the PM of muscle or adipocytes, respectively [8–10]. Insulin induces the repopulation of GSV, effectively promoting GLUT4 traffic from cellugyrin-containing to sortilin-containing vesicles, and increases the exocytic rate of GSV/IRV [13,20]. Insulin-derived signals regulate IRV exit from GSV before GLUT4 reaches the PM. Thus, insulin allows GLUT4 to escape its retention in 'idle' dynamic cycling between storage and recycling compartments (Figure 1).

The Static Pool Model. An alternative model suggests that the protein tether for the ubiquitin-like UBX domain for GLUT4 (TUG) links GLUT4 and IRAP to the Golgi matrix, and insulin leads to TUG proteolysis and GSV/RE cycling [21]. In this model insulin is thought to 'kick start' the exit of IRV from the GSV towards the PM, from whence subsequent cycles of GLUT4 internalization and re-externalization occur through the RE directly. Other models do not invoke the need for physical tethers and instead entertain the concept that insulin-derived signals cause the budding of vesicles from a non-recycling pool within the TGN/endosomes that then traffic towards the RE to exit through the recycling pathway. These models are incorporated in Figure 1. Of note, GLUT4 storage and cycling might differ in human skeletal muscle where GSV biogenesis appears to require clathrin22, which is not expressed in rodents [22].

While it is not yet possible to reconcile every aspect of these models, a common practice to distinguish exocytosis from the recycling versus the specialized compartment is to compare basal recycling with insulin-stimulated exocytosis. Manipulations that alter the latter but not the former are suggested to act on the specialized compartment (GSV and/or IRV). From this perspective one can propose that most insulin-dependent exit of GLUT4 is directly from the specialized compartment, given the requirement for VAMP2 in the insulin-dependent gain in GLUT4 at the PM, as occurs in muscle cells [15]. In adipocytes, the arrival near the membrane of small (*ca* 60 nm diameter) and large (*ca* 150 nm diameter) vesicles suggests that both GSV and RE might be their source [23] (Figure 1).

Insulin-Derived Signals Regulating Intracellular GLUT4 Traffic: The Akt- and Rac-Signaling Arms

Following insulin binding and autophosphorylation of its receptor, there is rapid phosphorylation of insulin receptor substrate (IRS) 1 and IRS2. Both IRS1 and IRS2 lead to phosphatidylinositol 3-kinase (PI3K) activation but only IRS1 is required for signal transmission towards GLUT4 mobilization [24]. Diacylglycerol kinase-ζ binds IRS1 in the basal state and dissociates in response to insulin, allowing GLUT4 translocation to proceed [25]. PI3K activation constitutes a point of bifurcation of insulin signaling, activating two separate cascades typified by activation of the serine/threonine kinase Akt and the Rho-family small GTPase Rac1, which in parallel promote GLUT4 translocation [24] (Figure 2).

In skeletal muscle Rac1 inhibition does not affect Akt and Akt inhibition does not affect Rac1, while each partially decreased insulin-stimulated glucose transport and joint inhibition annulled this response [26]. Although most evidence shows independent activation of Rac1 and Akt downstream of PI3K [26,27], crosstalk between these pathways may also occur. In one study Akt inhibition/silencing reduced Rac1 activity [28], while overexpression of constitutively active Akt can cause Rac1 activation [28,29] and Rac1 superactivation can activate Akt [30].

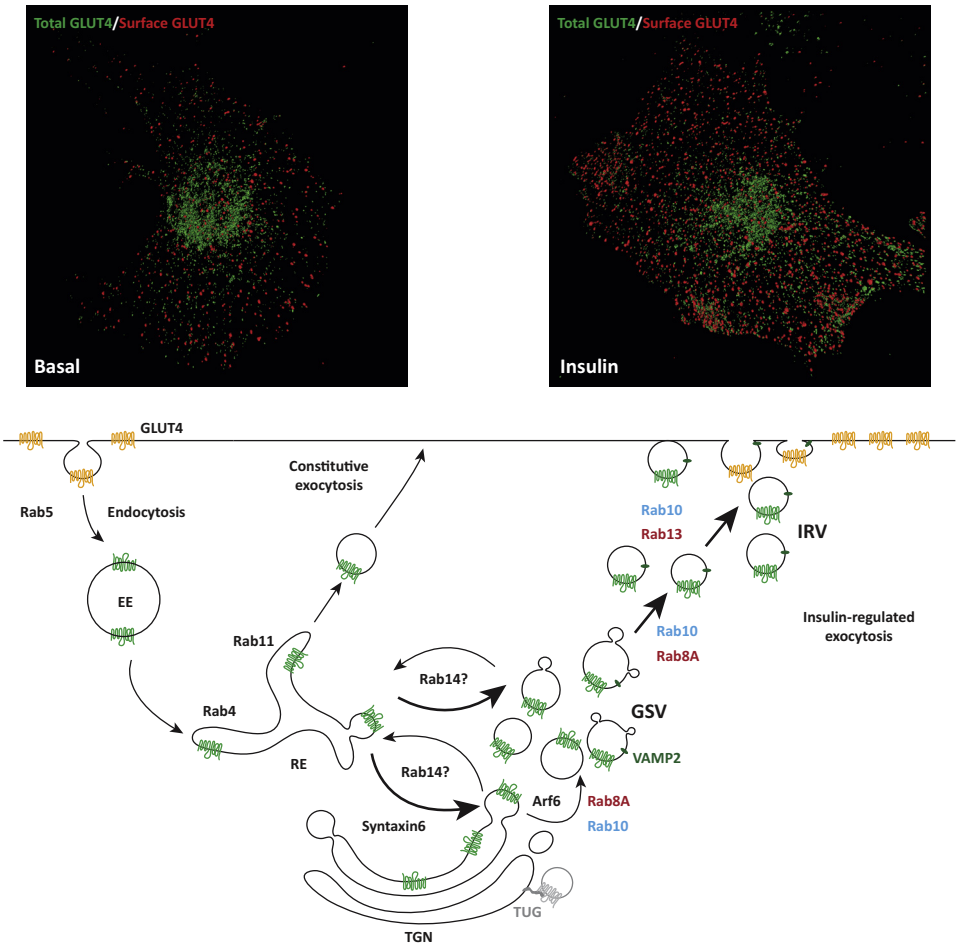

Figure 1. GLUT4 is Highly Compartmentalized and Undergoes Continuous Cycling to and from the Plasma Membrane (PM). Under basal conditions GLUT4 recycles constitutively through endocytosis and exocytosis as it moves from the PM to the early endosomes (EE) and from the EE to the recycling endosomes (RE) before returning to the PM. In addition, there is dynamic sorting of GLUT4 from the RE and trans-Golgi network (TGN) to generate glucose storage vesicles (GSV). Following insulin stimulation an increase in GLUT4 translocation to the PM occurs through the recruitment of GSV-derived insulin-responsive vesicles (IRV). The insets depict renderings of intracellular and surface GLUT4 under basal and insulin-stimulated conditions based on images acquired using super-resolution microscopy. L6 myoblasts transfected with GFP-GLUT4-myc were stained without permeabilization with anti-myc antibody to detect surface GLUT4. The GFP (green) signal represents all GLUT4 while the myc signal (red) represents PM-inserted GLUT4. The schematic representation of GLUT4 compartments reflects these color differences. Rab GTPases associated with each step of vesicular translocation and vesicle budding are depicted, as described in the text, to accommodate predictions of various models including the static model of retention regulated by the cleavage of tether for the ubiquitin-like UBX domain for GLUT4 (TUG). VAMP2 is a cargo protein and fusogen of the insulin-responsive vesicles. Rab GTPases mediating the traffic directionality of each step are indicated and discussed in the text.

Interestingly, overexpression of Engulfment and Cell Motility Protein 2 (Elmo2), a known Rac1 activator, did not cause Akt activation but promoted its membrane compartmentalization, resulting in enhanced insulin-stimulated GLUT4 translocation [31].

The Akt Signaling Arm Leads to Activation of Rab GTPases, Key Regulators of GLUT4 Traffic

Akt Regulation of Rab GAPs. Akt binds to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) produced by PI3K at the PM and intracellular vesicles, where it is phosphorylated by

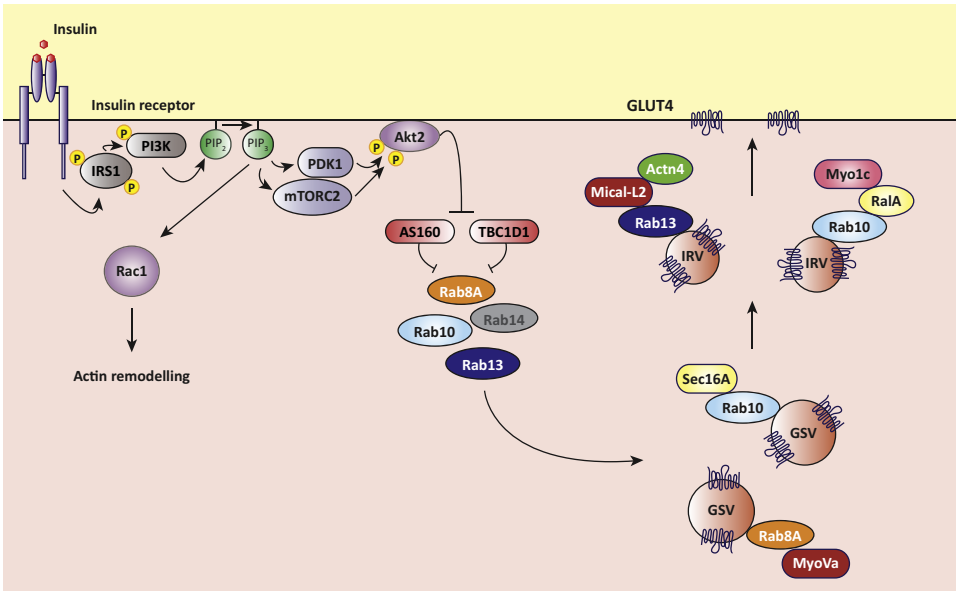

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Figure 2. Canonical and Novel Signals in Insulin-Stimulated GLUT4 Translocation. Following insulin binding and autophosphorylation of the insulin receptor, a cascade of phosphorylation events, mediated through insulin receptor substrate 1 (IRS1) and phosphatidylinositol 3-kinase (PI3K), result in the association of Akt with phosphatidylinositol 3,4,5-trisphosphate (PIP_3) at the plasma membrane (PM), where it is activated by phosphatidylinositol-dependent kinase 1 (PDK1) and mTORC2. Activated Akt phosphorylates and consequently inhibits the Rab GTPase-activating proteins (GAPs) AS160/TBC1D4 and TBC1D1, removing their inhibitory action on their target Rab GTPases. Through a variety of effects on vesicular traffic, Rab GTPases coordinate the translocation of GLUT4 to the PM. White letters identify Rab GTPases mediating GLUT4 translocation in muscle cells and black letters identify those involved in adipocytes. Rac1 is also activated by PI3K, and its actin remodeling action contributing to GLUT4 translocation is depicted in Figure 3.

phosphatidylinositol-dependent kinase (PDK) and mTORC2 on Th308 and S473, respectively. Activated Akt phosphorylates the Rab GAP Akt substrate of 160 kDa, also known as TBC1D4 (TBC1D4/AS160), and its family member TBC1D1 [24]. Although TBC1D4/AS160 and TBC1D1 are both Rab GAPs, with similar downstream targets *in vitro*, they have nonredundant functions towards GLUT4 translocation [32]. Under basal conditions TBC1D4/AS160 and TBC1D1 on GLUT4-containing vesicles attenuate GTP loading of their target Rab GTPases to prevent GLUT4 translocation (Figure 2). Recently, TBC1D13 was also found to inhibit insulin-stimulated GLUT4 translocation, as do its aforementioned family members [33]. Phosphorylation by Akt results in TBC1D4/AS160 and TBC1D1 inactivation, precluding their inhibitory effect on Rabs and promoting GLUT4 translocation [24].

Rab GTPases: A Crux between Signal Transduction and Vesicle Traffic. Rab GTPases are key determinants in the generation and/or destination of all events in intracellular traffic. In muscle cells Rab8A and Rab13 are downstream targets of TBC1D4/AS160, whereas Rab14 appears to be more stringently regulated by TBC1D1 [24]. In adipocytes Rab10 is the major GTPase downstream of TBC1D4/AS160. The recently identified phosphotyrosine-binding domain in TBC1D4/AS160 enables phospholipid binding and is proposed to facilitate GSV interaction with the PM [34], supporting a dual action of TBC1D4/AS160 on vesicles and at the periphery. The gain in surface GLUT4 resulting from experimental silencing of TBC1D4/AS160 or TBC1D1 is partly restored by concomitantly silencing each of these Rab GTPases [24].

Rab8A: Rab8A is required for the net process of GLUT4 translocation in muscle cells, based on gain- and loss-of-function experiments [35]. Mechanistically, insulin promotes Rab8A binding

to MyoVa, and interaction of MyoVa with actin filaments is required for GLUT4 translocation. Rab8A and MyoVa colocalize with GLUT4 in the perinuclear region and loss of MyoVa tightly clusters GLUT4 in this region, suggesting that the motor protein is required for processive exit of the vesicles from this location [36]. In L6 myoblasts and adipocytes, GLUT4 vesicles containing Rab8A do not reach the area just beneath the PM visualized with TIRFM [36], although they appear to do so in C2C12 myoblasts [37]. Nonetheless, additional proteins are required to regulate submembrane events.

Rab13: We recently identified the scaffold protein Mical-L2 as an effector of insulin-activated Rab13 in muscle cells. GLUT4, Rab13, and Mical-L2 colocalize in punctate structures near the PM. Rab13 binding unfolds Mical-L2 exposing a binding site for the filamentous cortical protein α-actinin-4 (Actn4), enabling tethering of GLUT4 vesicles to cortical actin filaments as an obligatory preamble to vesicle fusion [38]. The perinuclear action of Rab8A and peripheral action of Rab13 in muscle cells confirm the dual action of TBC1D4/AS160 in those locations [39].

Rab10: Rab10 is the major Rab GTPase implicated in GLUT4 exocytosis in adipocytes [40–44], and being in the same family as Rab8A and Rab13 it potentially mediates the function that those two Rabs have in muscle cells. Moreover, Rab10 may also bind MyoVa in adipocytes but at peripheral regions of the cell [42]. A compelling effector of Rab10 in adipocytes is Sec16A, acting at the exit point of intracellular compartments involved in insulin-regulated GSV formation [45]. In mice Sec16A knockdown phenocopied Rab10 knockdown in blunting insulin-dependent GLUT4 translocation [45]. In addition, Rab10 interacts with Exoc6, a subunit of the exocyst, to promote GLUT4 vesicle fusion with the PM [43] and activates RalA, an exocyst-interacting GTPase required for GLUT4 translocation through interaction with Myo1c [46,47]. Rab10 induces GTP loading of RalA through its guanine nucleotide exchange factor (GEF) Rlf/Rgl2, and through loss-of-function strategies these proteins were deemed to be required for insulin-stimulated glucose uptake [47]. Interestingly, a RalA GAP complex (RGC) inhibits RalA GTP loading. RGC activity is inhibited by insulin through Akt2 phosphorylation of its RGC2 subunit [48]. Recently, it was reported that activated RalA binds to Sec5 and Exo84, members of the exocyst complex that tethers GLUT4 vesicles to the PM. Intriguingly, the kinase TBK1, which mediates inflammatory responses, was found to phosphorylate Exo84 resulting in GLUT4 vesicle release to proceed to fusion [49].

Rab14: Recent findings suggest that Rab14 mediates GLUT4 replenishment of GSV or the TGN from sorting endosomes [42,50,51]. Others localize Rab14 to transferrin-positive compartments, indicating that Rab14 is involved in GLUT4 movement in endosomes [41].

Other Rab GTPases: In addition to these three Rabs, which are well established as regulators of GLUT4 traffic, Rab28 was recently described as becoming GTP loaded in response to insulin, possibly acting downstream of TBC1D4/AS160 and TBC1D1. Rab28 knockdown in muscle diminished basal glucose uptake while in adipocytes constitutively active Rab28 elevated it, suggesting involvement of the GTPase in GLUT4 traffic [52]. Finally, a screen for the effects of overexpressing a battery of TBC1D/Rab GAP proteins identified TBC1D13 as a possible modulator of GLUT4 traffic, with Rab35 as its downstream mediator [33]. Although not substrates of TBC1D4/AS160 *in vitro*, Rab4 and Rab11, which mediate forward traffic of vesicles in the RE, are activated in response to insulin, whereas the retrograde traffic regulator Rab5a of early endosomes is inhibited. Chronic-insulin-induced insulin resistance blocks GLUT4 exit from the Rab5-positive endosomes towards SE and storage compartments [53]. Rab4 mediates insulin-stimulated GLUT4 translocation and its interaction with syntaxin4 had suggested potential involvement in both GSV sorting and fusion [9,10]. However, recent work shows that Rab4 is involved in endosomal traffic of GLUT4 and not directly in exocytosis

[42]. Rab11 participates in GLUT4 sorting to GSV and the Rab11-interacting protein Rip11 forms a complex with TBC1D4/AS160 that may regulate the traffic of GLUT4 in response to insulin [10,54].

The Rac1 Signaling Arm Leads to Cortical Actin Remodelling

As mentioned above, downstream of PI3K, and in parallel with Akt activation, insulin activates the Rho-family small GTPase Rac1. Rac1 is a major regulator of actin filaments at the cell cortex. Interestingly, insulin and muscle contraction stimulate Rac1 activation of actin remodeling, glucose uptake, and GLUT4 translocation in muscle cells and mouse skeletal muscle [55–57] (see Box 1 for the Rac1 response to muscle contraction). In muscle a GEF for Rac1, FLJ00068, was also implicated in insulin-stimulated GLUT4 translocation [58,59]. Other Rho-family GTPases are possibly also involved in GLUT4 traffic in adipocytes [60]. While the contribution of Rac1 to insulin-stimulated glucose uptake was initially questioned, more recently the enhanced insulin-stimulated GLUT4 translocation induced by P-Rex1, a Rac1 GEF, verified Rac1 input into GLUT4 translocation [61].

Consistent with Rac1 activation, insulin induces cortical actin filament branching, and interfering with filament remodeling reduces GLUT4 translocation [27] and glucose uptake [56,62]. Silencing or inhibiting Rac1 reveals that active remodeling, rather than preformed actin filaments, is required for GLUT4 translocation (Figure 3). The remodeled cortical actin network of muscle cells serves as a scaffold for insulin-derived signals and GLUT4 vesicles [63] and appears to coincide with areas of GLUT4 insertion [63]. In adipocytes actin dynamics contributes to tethering, docking, and/or fusion of GLUT4 vesicles [64].

Rac1-Dependent Effectors Leading to Actin Remodeling. Rac1 activates the nucleating factors WAVE and neuronal Wiskott–Aldrich syndrome protein (n-WASP). n-WASP, through interaction with various effectors, regulates GLUT4 endocytosis [65] and translocation [66,67]. n-WASP and WAVE activate the branching complex Arp2/3, and silencing components of the latter reduces insulin-induced actin remodeling and GLUT4 translocation [27,68]. Moreover, cortical actin branching activates the phosphatase slingshot and its downstream target cofilin,

Box 1. Rac1 Input into Contraction-Induced GLUT4 Traffic in Muscle

Muscle contraction is a major physiological stimulus of GLUT4 translocation. It is independent of insulin and the source of the GLUT4 vesicles mobilized and mechanisms involved are distinct for each stimulus [97]. While insulin promotes largely GLUT4 vesicle exocytosis, muscle contraction and its downstream signals also reduce GLUT4 retrieval from the membrane via endocytosis [98–101]. Interestingly, exercise also improves insulin sensitivity in muscle, as has been summarized recently by others [102].

A large body of work has examined the partial contributions of AMPK, Ca^{2+}, calmodulin-sensitive kinases, and protein kinase C isoforms to contraction-stimulated glucose uptake [97,102] and is not reviewed here. These signals may synergize or act in series [103] to produce the full response of GLUT4 translocation and may explain the failure to identify a sole signaling event required for contraction-stimulated glucose uptake, as these pathways may be redundant and may compensate for one another when silenced.

Recently, Rac1 emerged as another important contributor to muscle-contraction-stimulated glucose uptake [104,105]. Mechanical tension is required for contraction-stimulated glucose uptake via Rac1 [103–105] and passive stretch *per se* also activates Rac1 [106,107]. The dystrophin–glycoprotein complex acts as mechanical sensor during contraction and stretch, with Src-family kinase recruitment initiating Rac1 activation [107,108].

Rac1 inhibition reduces contraction- and passive-stretch-stimulated glucose uptake in mouse skeletal muscle independent of AMPK and intracellular Ca^{2+} [57,104,105]. Accordingly, Rac1 knockout mice exhibit attenuated exercise-induced increases in surface GLUT4 in skeletal muscle. This suggests Rac1-mediated regulation of GLUT4 translocation to the sarcolemma [105]. As in the case of insulin stimulation, contraction-activated Rac1 appears to signal to the subcortical actin cytoskeleton (distinct from the sarcomeric filaments) in mature muscle, and accordingly cortical actin filament depolymerization attenuated contraction- and stretch-induced glucose uptake [57,104,105].

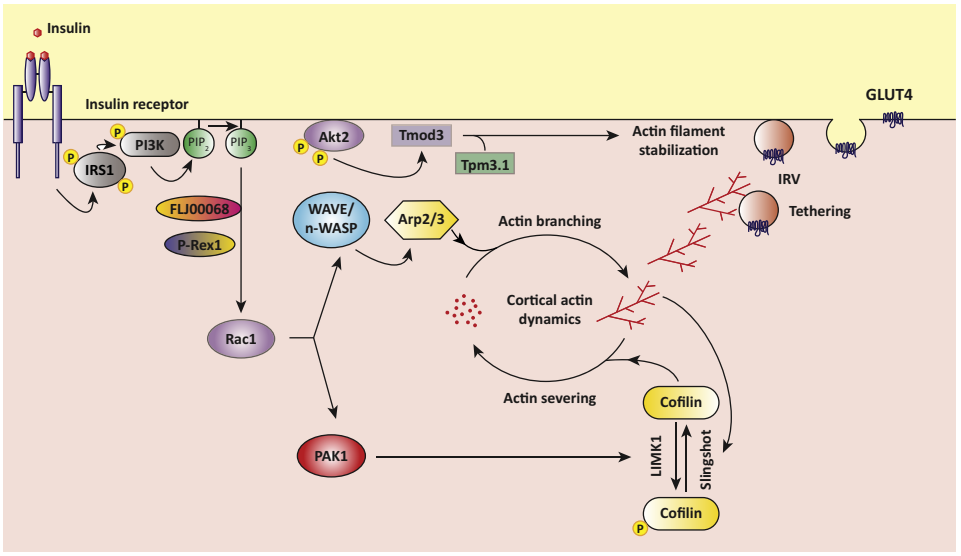

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Figure 3. Rac1 is Activated Downstream of Phosphatidylinositol 3-Kinase (PI3K) Activation, Potentially through the Actions of the Rac1 Guanine Nucleotide Exchange Factors (GEFs) FLJ00068 and P-Rex1. Rac1 in turn activates WAVE/neuronal Wiskott–Aldrich syndrome protein (n-WASP)–Arp2/3 signaling, causing cortical actin filament branching. As filaments grow they activate the phosphatase slingshot that activates cofilin, which induces actin filament severing beneath the plasma membrane (PM). In parallel, activated Akt phosphorylates Tmod3, which interacts with Tpm3.1-positive cortical actin to stabilize actin filaments. These events are all required for GLUT4 vesicle tethering beneath the PM.

important for actin severing and the regeneration of free actin for further assembly [69]. Complementing this action, insulin-stimulated Rac1 activates LIM kinase to inhibit cofilin, resulting in an iterative cycle of actin branching and severing.

Insulin-stimulated Rac1 also activates the serine/threonine kinase PAK1 in muscle cells [68] and skeletal muscle [56]. PAK1 inhibition blunts insulin-induced cortical actin remodeling, cofilin activation, and GLUT4 translocation [70]. Moreover, diminished Rac1 and PAK1 activation in insulin resistance [56] constitutes the first functional defect in proteins involved in actin dynamics and highlights the physiological and pathological relevance of Rac1 signaling.

New findings suggest that Akt signalling also impacts actin dynamics by phosphorylating tropomodulin3 (Tmod3), which aids tropomyosin3.1 (Tpm3.1) in capping and stabilizing cortical actin filaments [71,72]. Knockdown of either Akt2 or Tmod3 results in abnormal actin remodelling within ~100 nm of the PM [72] (Figure 3).

GLUT4 Vesicle Tethering to Cortical Actin via Myo1c, MyoIIA, and Actn4

In addition to interacting with processive motors such as MyoVa that mediate GLUT4 vesicle displacement (see above), cortical actin also acts as a submembrane tethering platform for incoming vesicles. Insulin reduces the proportion of highly mobile GLUT4 vesicles within 100–200 nm of the PM (visualized by TIRFM illumination) of both adipose and muscle cells, presumably as a preamble to faithful vesicle fusion [67,73–75]. Considering that incoming GLUT4-containing vesicles can measure upwards of ~100 nm in diameter, these observations could reflect regulation of vesicle tethering, docking, and/or fusion. By contrast, in isolated muscle fibers a considerable number of GLUT4 vesicles lie immobile beneath the sarcolemma and T tubules before stimulation [76]. Various proteins have been identified as potential

regulators of GLUT4 movement and docking beneath the PM, in part by facilitating vesicle binding to actin filaments.

Myo1c. Myo1c and GLUT4 colocalize with actin filaments and silencing Myo1c reduces the gain in surface GLUT4 in adipocytes [77] and muscle cells [75]. Myo1c overexpression enhances the fraction of static vesicles beneath the PM and potentiates the insulin-dependent gain in PM-inserted transporters. Conversely, Myo1c knockdown or expression of an actin-binding-deficient Myo1c mutant accelerates GLUT4 vesicle mobility beneath the PM and abrogates GLUT4 insertion [75]. Besides GLUT4 vesicle tethering, Myo1c also contributes to the maintenance of proper actin dynamics by associating with Rictor to mediate paxillin phosphorylation, which regulates cortical actin remodeling [78]. Myo1c also associates with RalA, which, as mentioned above, engages the exocyst during insulin-stimulated translocation [43,46]. In skeletal muscle Myo1c overexpression elevates insulin-stimulated glucose uptake, whereas it is attenuated by a Myo1c mutant unable to hydrolyze ATP [79].

MyoIIA. The 'non-muscle' MyoIIA contributes to the organization and contraction of actin filaments. Inhibiting the MyoIIA activator myosin light chain kinase interferes with actin remodeling and GLUT4 translocation [80]. GLUT4 translocation to the PM precedes that of MyoIIA [81,82], suggesting that MyoIIA promotes GLUT4 insertion, probably by aiding in the formation of the VAMP2–syntaxin4 complex (discussed below). MyoIIA colocalizes with Tpm3.1-containing actin fibers near the PM, potentially recruiting MyoIIA while simultaneously restricting Myo1c binding [71]. We envisage that Myo1c contributes to GLUT4 vesicle tethering to cortical actin while MyoIIA–Tpm3.1 aides in cortical actin contractility leading to vesicle docking/fusion through SNARE complexes.

Actn4. Actn4 is an antiparallel, dimeric filamentous protein that interacts with cortical actin filaments. Notably, Actn4 and GLUT4 can directly bind *in vitro* [83], and in cells insulin promotes the association of Actn4 with GLUT4 at the actin-rich cortex [84]. Actn4 knockdown interferes with GLUT4 retention at the cell cortex effectively preventing GLUT4 insertion in the PM without affecting actin remodeling, suggesting that Actn4 may link IRV to actin structures [83]. Actn4 integrates cortical actin with Akt-derived signals, as Rab13 unfolds the linker protein Mical-L2 to expose an Actn4-binding site [38] as discussed above (Figure 3).

In addition to the actin cytoskeleton, microtubules also participate in GLUT4 traffic. Mechanistically, microtubule disruption rapidly prevents GLUT4 sorting from the PM back towards perinuclear GSV in L6 myoblasts and, consequently, insulin-dependent re-exocytosis is attenuated [19]. These findings suggest that microtubules are necessary for retrograde GLUT4 traffic. In addition, new findings reveal that insulin regulates microtubule polymerization near the PM, increasing microtubule density and curvature [85]. Furthermore, GLUT4 vesicles displace linearly and parallel with the PM along microtubules [73] and fusion events occur preferentially close to microtubules [85], although the underlying mechanism requires molecular definition.

GLUT4 Vesicle Fusion with the PM

Once GLUT4 vesicles arrive and tether at the cell periphery they undergo fusion with the PM. This process is mediated by SNARE proteins (canonical fusogens) and several regulatory factors [86]. Before membrane fusion, SNAREs exist in monomeric conformations on the incoming and target membranes, and fusion is driven through the irreversible assembly of one v-SNARE with two target-membrane SNAREs (t-SNAREs). These form a ternary complex that brings their respective lipid bilayers into sufficient proximity for thermodynamic melting [87]. The t-SNAREs involved in GLUT4 exocytosis are syntaxin4 and SNAP23 on the PM and the v-SNARE is VAMP2 on the approaching vesicle [86]. Some promiscuity has been observed between VAMP2, VAMP3, and VAMP8 for t-SNARE complex assembly in adipocytes, but most

evidence points to VAMP2 mediating GLUT4 vesicle fusion [8]. Syntaxin4 and SNAP23 suffice for vesicle tethering to the PM while VAMP2 promotes fusion [88]. In muscle cells VAMP2 but not VAMP3 is the v-SNARE responsible for the insulin-responsive gain in surface GLUT4, while VAMP7 mediates the fusion of constitutively recycling vesicles [15].

GLUT4 vesicle fusion is regulated through Munc18c, Synip, the calcium sensor Doc2b, and its regulator Tctex1d2, which determine syntaxin4 availability and SNARE complex formation. A recent model suggests the existence of two syntaxin4 pools in adipocytes, one associated with SNAP23 mediating constitutive recycling and another with VAMP2 and Munc18c that is engaged with insulin stimulation [89]. A second model proposes that Munc18c is an inhibitory factor and that under basal conditions it is bound to syntaxin4, precluding its availability for SNARE complex assembly. According to this model, Munc18c is phosphorylated directly by the activated insulin receptor, thereby dissociating from syntaxin4 and allowing SNARE complex assembly and GLUT4 vesicle fusion with the PM. Conversely, Munc18c is dephosphorylated by PTP1B, re-associating with syntaxin4 and inhibiting glucose uptake [90]. Accordingly, mice depleted of PTP1B or with PTP1B inhibition exhibit elevated glucose uptake and enhanced glucose homeostasis [91], although clearly this could be the consequence of the alteration of other reactions normally regulated by the phosphatase. Intriguingly, Munc18c expression is reduced in the adipose tissue of morbidly obese individuals and in high-fat-fed mice, potentially to compensate for underlying compromised GLUT4 vesicle fusion [90,92].

Like Munc18c, Synip binds syntaxin4 under basal conditions, precluding its interaction with VAMP2, SNARE complex assembly, and vesicle fusion. *In vitro* studies show Synip binding to syntaxin4 and preformed syntaxin4-SNAP23 dimers, inhibiting docking and SNARE-

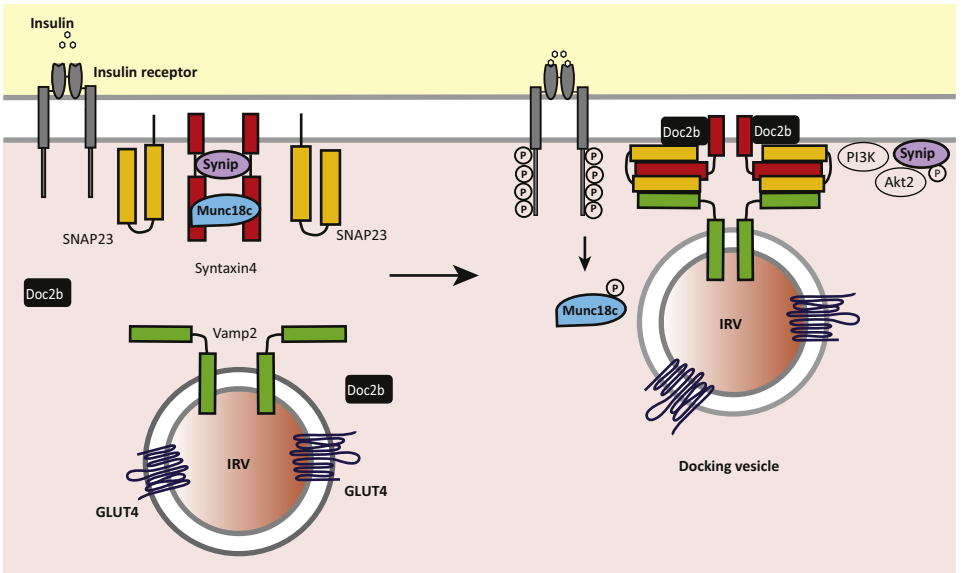

Figure 4. Membrane Fusion by SNAREs and Their Regulators. Under basal conditions SNARE proteins [VAMP2 on the vesicle and the target-membrane SNAREs (t-SNAREs) syntaxin4 and SNAP23 on the target plasma membrane (PM)] are unengaged. Munc18c and Synip are bound to syntaxin4, preventing SNARE complex assembly. With insulin stimulation the insulin receptor phosphorylates Munc18 and Akt phosphorylates Synip, both processes releasing syntaxin4 and promoting SNARE complex assembly. Insulin-stimulated Ca^{2+} transients recruit Doc2b to the PM to bind syntaxin4 in a Ca^{2+}-dependent manner, enabling SNARE complex assembly and GLUT4 vesicle fusion with the PM.

Box 2. Participation of Ca^{2+} in Insulin-Stimulated GLUT4 Traffic

The participation of Ca^{2+}-sensitive regulators in GLUT4 vesicle fusion suggests a role for Ca^{2+} in insulin action, whether as a signal or as a required local element. In skeletal muscle Ca^{2+}-sensitive fluorescent dyes detect insulin-dependent increases in Ca^{2+} in the proximity of the PM, thought to be mediated by influx through membrane channels [109]. In skeletal and cardiac muscle cells in culture, cytosolic Ca^{2+} spikes occur as a result of both Ca^{2+} entry [98] and insulin-induced sarcoplasmic reticulum Ca^{2+} release. The latter is released through IP_3-ligated channels and possibly ryanodine receptor channels [110–112]. Diverse strategies to dampen intracellular Ca^{2+} reduce insulin-mediated GLUT4 translocation. Mechanistically, Ca^{2+} is required to achieve full stimulation of Akt and is likely to directly impact vesicle fusion [98,110,113]. Additionally, ionophore-mediated, sustained Ca^{2+} influx *per se* promotes GLUT4 exocytosis and reduces its endocytosis [99].

SNARE complexes mediating GLUT4 vesicle fusion are regulated by the Ca^{2+}-dependent proteins Doc2b, Tctex1d2, and E-Syt1 [8]. Doc2b and Tctex1d2 regulate syntaxin4 availability for SNARE complex formation and GLUT4 vesicle fusion [114]. The low-level, submembrane, and transient Ca^{2+} increases are compatible with the Ca^{2+} sensitivity of Doc2b. The latter contains a munc13-interacting domain and C2A/C2B domains that bind phospholipids in a Ca^{2+}-dependent manner [115]. The protein interactions and mechanism of action of Doc2b remain controversial. Doc2b is reportedly PM associated and binds to phosphorylated Munc18c in response to insulin, enabling SNARE complex formation. However, Doc2b is also visualized on small cytosolic structures and recruited to the PM with insulin stimulation, where it binds syntaxin4 in a Ca^{2+}-dependent manner facilitating membrane curvature and SNARE assembly [114,116]. Whatever its location or partner, Doc2b appears to facilitate vesicle fusion. Accordingly, isolated adipocytes from Doc2b-null mice show reduced GLUT4 translocation [117].

The protein Tctex1d2 counteracts Doc2b binding to syntaxin4, reducing GLUT4 vesicle fusion. Apparently, insulin stimulation increases Tctex1d2–syntaxin4 binding at the expense of the Doc2b–syntaxin4 association, with consequent SNARE complex formation [118]. Finally, the protein E-Syt1, when phosphorylated by cyclin-dependent kinase-5 with insulin stimulation, associates with GLUT4 and, in a still unknown way, enables insulin-stimulated glucose uptake in adipocytes [119]. The similarity between E-Syt1 and synaptotagmins suggest that this protein might mediate a Ca^{2+}-dependent input towards SNARE complex formation, in this case responding to Ca^{2+} levels higher than those sensed by Doc2b.

Outstanding Questions

What are the defining proteins of the GLUT4 storage compartment and the GLUT4 vesicles reaching the membrane that regulate the segregation and traffic of the transporter in these distinct vesicular compartments?

What are the GEFs activating Rab8a and Rab13?

Do Rab10 and Rab13 regulate GLUT4 vesicle tethering, since they engage putative tethering complexes in adipocytes and muscle cells, respectively?

Since actin remodeling defines the loci of GLUT4 tethering and release for fusion, what are the molecular inputs of MyoIIA and Myo1c and how are they affected by Tpm3.1 and Tmod3?

Does Rac1 activation have input beyond triggering actin cytoskeleton remodeling? What is the mechanism of PAK1 participation in GLUT4 traffic?

Is syntaxin4 availability for SNARE complex formation regulated by Ca^{2+} in response to insulin? Can GLUT4 vesicles release Ca^{2+} to modulate the local environment?

Is Ca^{2+} a signal or only a permissive factor in insulin-stimulated GLUT4 vesicle tethering to the PM? Does Ca^{2+} regulate steps in GLUT4 translocation additional to SNARE complex formation?

What specific steps in GLUT4 translocation are defective in obesity-induced insulin resistance and diabetes?

dependent fusion [93]. PI3K and Akt activation by insulin appears to exert dual regulation of Synip, as the latter binds to PIP_3 [94] and is phosphorylated by Akt [95]. Synip phosphorylation allows dissociation from syntaxin4, promoting GLUT4 translocation, SNARE complex assembly, and glucose uptake [95,96]. Collectively these studies suggest that both Munc18c and Synip act as negative regulators of GLUT4 vesicle fusion and their blocking action is eliminated by insulin-dependent signals (Figure 4). However, while Munc18c inhibits the complex formed between t-SNAREs (syntaxin4–SNAP23), Synip might inhibit the final SNARE complex assembly. Last, several Ca^{2+}-sensitive proteins, including double C2-like domains beta (Doc2b), Tctex1 domain containing 2 (Tctex1d2), and extended synaptotagmin-like protein with five C2 domains and transmembrane domain (E-Syt1), regulate SNARE complex formation. As localized or transient Ca^{2+} changes occur in response to insulin, subsequent activation of these Ca^{2+}-sensitive proteins may regulate GLUT4 vesicle fusion with the PM (Box 2).

Concluding Remarks and Future Perspectives

Notable advances have been made in the past few years in our understanding of GLUT4 traffic. New protein constituents of the GSV have been identified, demanding future examination of their function. Significant discoveries have also been made in insulin signals proximal to GLUT4 traffic, with the identification of two new substrates of Akt2 in adipocytes: RGC2, a subunit of the RalA GAP; and Tmod3, a regulator Tpm3.1-decorated actin filament dynamics. These findings place Akt2 input at a regulatory node distal to the receptor impacting GLUT4 vesicle traffic. Major developments have arisen regarding Rab GTPase function, with the identification of the Rab10 effectors Sec16A and Exoc6 in adipocytes and the Rab13 effector Mical-L2 in muscle cells. The latter two effectors may regulate GLUT4 vesicle tethering. It remains to be established whether there is cellular specificity for the action of each Rab GTPase. Additionally, Rab28 and Rab35 emerge as potential targets of Rab GAP proteins that contribute to GLUT4 vesicle traffic.

TIRFM has illuminated details in GLUT4 vesicle tethering and fusion as well as cortical actin filament remodeling and the function of myosin motors. Our molecular understanding of actin dynamics and its pivotal role in GLUT4 traffic has grown, but its precise consequences for GLUT4 vesicle tethering and docking requires further scrutiny. Remodeled actin orchestrates interactions between signaling molecules, tropomyosin/tropomodulin, and Myo1c and MyoIIA around the GLUT4 vesicle as its approaches the cell surface. Governing actin remodeling, the contribution of Rac1 to GLUT4 traffic has been cemented, as recent work demonstrates its necessity for insulin- and contraction-stimulated glucose uptake and GLUT4 translocation in mouse skeletal muscle.

GLUT4 vesicle fusion has been further scrutinized, and a picture emerges whereby insulin-induced phosphorylation of Munc18c and Synip frees up SNARE proteins for vesicle docking and fusion. New discoveries also reveal regulation of Munc18c through the Ca^{2+}-sensing proteins Doc2b and E-syt1, and interestingly insulin evokes intracellular Ca^{2+} transients. Thus, Ca^{2+} emerges as a regulatory element in the final step of GLUT4 vesicle fusion.

Every newly discovered node of insulin regulation has the potential to yield an antidiabetic therapy that targets muscle/fat glucose transport. In this regard, defects in Rac1, PAK1, Munc18c, and Tpm3.1 have been linked to metabolic dysregulation. New efforts should continue to reveal mechanisms of control and their potential failure in insulin resistance and diabetes in an attempt to identify therapeutic targets.

Overall, our understanding of GLUT4 vesicle translocation from budding to the PM and tethering, docking, and fusion has progressed significantly. Advanced single-particle tracking analysis and super-resolution microscopy, as well as innovations in CRISPR/Cas9 gene editing, may be applied in the future to examine GLUT4 traffic in primary adipocytes and skeletal muscle fibers.

Further open questions on GLUT4 translocation are outlined in the Outstanding Questions.

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