Insulin-stimulated endosomal trafficking of GLUT4: a change to the model?

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Summary statement:

The authors re-examine the delivery of GLUT4 vesicles to the surface of fat and muscle cells based on new studies suggesting that heterotypic fusion with endosomes may play a more important role than previously realized.

Abstract

Recent advances in spatiotemporally resolved imaging and single-molecule labeling technologies have provided new mechanistic insight into the very early phases of insulin-responsive GLUT4 trafficking. Live-cell assays combining quantum dot tracking and fluorescence-based fusion reporters have uncovered a previously overlooked, insulin-induced initial and fundamental step: static GLUT4 vesicles undergo heterotypic fusion with transferrin receptor-positive endosomes. This insulin-induced fusion functions as a molecular gateway – termed "Fusion-Guided GLUT4 Entry" (FGG4E) – that enables GLUT4 molecules, originally sequestered in static vesicles, to circulate within a dynamic endosomal network when insulin is present, escaping the trafficking itinerary that leads to static retention. Through this pathway, insulin-stimulated GLUT4 is efficiently delivered to the plasma membrane and continues to recycle dynamically between the plasma membrane and endosomal compartments. After insulin withdrawal, GLUT4 molecules are retrieved from the endosomal system and returned to the static pool. In this Opinion article, we propose that this revised model highlights a key regulatory role for heterotypic vesicle fusion as a gateway linking basal retention with dynamic mobilization and recycling and redefines GLUT4 trafficking beyond the classical view of vesicle mobilization.

Introduction

A major consequence of insulin action is a dramatic increase in the rate of glucose transport into fat and muscle (Richter et al., 2025; Saltiel, 2021; Santoro et al., 2021) achieved by the regulated trafficking of the facilitative glucose transporter GLUT4 from insulin-sensitive intracellular stores to the plasma membrane (PM) (Bogan and Kandror, 2010; Bryant et al., 2002; Gould et al., 2020). It is well established that the insulin-regulated delivery of GLUT4 to the PM is impaired in insulin resistance and Type-2 diabetes mellitus (T2DM). Individuals with T2DM exhibit impaired insulin-stimulated glucose transport in muscle and fat (Richter et al., 2025; Santoro et al., 2021), and many studies have supported the hypothesis that GLUT4 sorting and/or trafficking are impaired in these individuals (Doehner et al., 2010; Pedersen et al., 1990; Richter et al., 2025; Sylow et al., 2021). For example, GLUT4 is mis-sorted into a denser membrane fraction in muscles from individuals with T2DM compared to those without the disease and insulin does not promote mobilization of GLUT4 from this fraction to the PM. Similarly, defective GLUT4 sorting has been described in adipocytes isolated from individuals with T2DM (Garvey et al., 1991; Kahn, 1996; Sinha et al., 1991) and in individuals with gestational diabetes (Garvey et al., 1993; Maianu et al., 2001).

Several early studies considering the nature of the intracellular GLUT4 compartment(s) were key to our understanding of GLUT4 trafficking, demonstrating that GLUT4 is distributed between two intracellular pools: transferrin receptor-(TfR) containing endosomes and a compartment that excludes TfR (Johnson et al., 1998; Lampson et al., 2000; Livingstone et al., 1996; Martin et al., 1998; Martin et al., 1996; Subtil et al., 2000). These studies provided a foundational framework to understand intracellular GLUT4 trafficking.

In the absence of insulin, GLUT4 is sequestered intracellularly by the coordinated action of two intracellular trafficking pathways (Karylowski et al., 2004; Martin et al., 2006; Proctor et al., 2006; Shewan et al., 2003; Zeigerer et al., 2002). The first operates between the PM and recycling endosomes and serves to efficiently internalize GLUT4 (Lampson et al., 2001; Subtil et al., 2000; Zeigerer et al., 2002). GLUT4 is then sorted from this pathway into a second cyclical pathway that operates between recycling endosomes, the *trans* Golgi network (TGN) and other vesicles and tubular structures (Lampson et al., 2001; Shewan et al., 2003; Zeigerer et al., 2002). The recycling of GLUT4 between the PM and these intracellular compartment(s) in the absence of insulin is as a result slow – far slower than (for example) the recycling of the TfR (Brewer et al., 2014; Govers et al., 2004; Habtemichael et al., 2011; Karylowski et al., 2004; Subtil et al., 2000). Consequently, GLUT4 is retained intracellularly by recycling through numerous compartments of the endosomal system. These compartments, together with the

GLUT4-positive regions of the TGN and other vesicles/tubules, are collectively referred to as the 'GLUT4 storage compartment' (GSC) (Gould et al., 2020). The major action of insulin is to alter these pathways to achieve increased PM levels of GLUT4. The intracellular compartment from which GLUT4 is delivered to the PM in response to insulin is usually referred to as 'insulin-responsive vesicles' (IRVs).

Considerable evidence supports the notion that adipocytes and muscle contain this unique IRV compartment to which GLUT4 is sequestered; this compartment might be present in all cells but is particularly well defined in insulin-sensitive tissues; see (Bryant and Gould, 2020; Gould et al., 2020). Though there is little debate that the main action of insulin is to drive increased levels of GLUT4 at the PM, recent data has led us to question the notion of how this is achieved: are IRVs directly delivered to the PM, or could the situation be more complex and involve insulin-dependent regulation of sub-endosomal GLUT4 trafficking?

Recent work from our labs and others suggests that the effect of insulin may encompass an insulin-stimulated liberation of GLUT4-containing vesicles underpinned by an increase in heterotypic fusion between these liberated vesicles with elements of the endosomal system (Hatakeyama and Kanzaki, 2011; Hatakeyama et al., 2022; Hatakeyama et al., 2019). This mechanism consequently delivers increased GLUT4 within the recycling endosomes which then reaches the PM. In this Opinion article, we discuss this recent evidence and evaluate whether it could provide a mechanism to fine-tune GLUT4 levels at the PM. We further suggest that this mechanism, acting in tandem with other regulatory pathways, might be particularly important under physiological conditions where circulating insulin concentrations fluctuate over narrower windows than those typically employed in experimental settings, and could play a significant role in specific tissues, such as fat and skeletal muscle.

Endosomes and GLUT4: A complex relationship

The advent of kinetic analyses and GFP-tagging studies led to the development of two mutually exclusive models to explain the marked intracellular sequestration of GLUT4 in the absence of insulin. The first model, 'dynamic retention', postulated that GLUT4 exchanges between the PM and intracellular compartments, including IRVs: in the absence of insulin, slow exocytosis from the GSC coupled with efficient and rapid endocytosis from the PM maintains the characteristic intracellular distribution of GLUT4. Insulin then induces a rapid increase in exocytosis of the IRVs, driving increased PM GLUT4 levels. The second model, 'static retention', posited a key difference: the IRVs do not cycle through the PM but rather are mobilized to the PM in response to an insulin stimulus. In this model, insulin increases PM

GLUT4 levels predominantly by promoting exocytosis of specialized vesicles, though an increased level of GLUT4 in recycling endosomes also contributes (**Fig. 1**). For details, see (Coster et al., 2004; Govers et al., 2004; Habtemichael et al., 2011; Karylowski et al., 2004; Martin et al., 2006; Muretta and Mastick, 2009; Muretta et al., 2008).

Most studies of GLUT4 trafficking have relied upon observations of the recycling of the reporter HA-GLUT4-GFP. This molecule contains an HA-epitope tag in the exofacial loop of GLUT4, which can be used to detect and/or label PM GLUT4 and thus study its recycling kinetics. By coupling these kinds of recycling experiments with the power of flow cytometry, Muretta et al. revealed a complex pattern of GLUT4 behaviour (Muretta and Mastick, 2009; Muretta et al., 2008). In the absence of insulin, around 75% of GLUT4 is packaged into non-recycling vesicles. Insulin was found to increase the amount of GLUT4 in the actively cycling pool ~5-fold and to increase the rate constant for exocytosis ~3-fold, providing compelling evidence in favour of a static retention model (**Fig. 1**). Importantly, however, these studies demonstrated that the recycling behaviour of GLUT4 was profoundly affected by the conditions of the experiment, including whether cells had been 'replated', and influenced by the sensitivity of the assay methods used in early studies (Muretta and Mastick, 2009; Muretta et al., 2008). Notably, the formation of a static and highly insulin-responsive GLUT4 retention system is also known to depend on cell differentiation status, with more mature adipocytes exhibiting enhanced sequestration of GLUT4 into specialized insulin-sensitive compartments (Romenskaia et al., 2025), reflecting the functional maturation of this regulatory system – a point we shall return to below.

Using a compartment-specific fluorescence quenching assay to look at GLUT4 distribution, McGraw and colleagues concluded that GLUT4 is distributed between two intracellular pools, one containing TfR and one not (Zeigerer et al., 2002). These pools were suggested to be in communication with one another and with the PM; importantly, insulin-stimulated GLUT4 delivery to the PM required both pools. The authors proposed a two-step model for GLUT4 trafficking in which the general endosomal recycling compartment plays a specialized role in the insulin-regulated traffic of GLUT4 (Zeigerer et al., 2002). First, they argue that GLUT4 is excluded from the PM by a retention-retrieval mechanism: based on single-cell assays, GLUT4 vesicles are five times more likely to fuse with endosomes than with the PM (Karylowski et al., 2004). Second, they argue that under their experimental conditions, an intracellular cycle of vesicle budding and fusion is an element of the active mechanism by which GLUT4 is retained.

Hence, depending on the cellular conditions and assays used, two models should be considered. In the first, GLUT4 is sequestered into a non-endosomal secretory compartment

which is selectively mobilized in response to insulin. In the second, GLUT4 is retained within endosomal-associated structures and insulin overcomes this retention, delivering GLUT4 into recycling vesicles and thence to the PM (**Fig. 1**). These distinct models need not be mutually incompatible, and it is important to note that elements of both may exist within insulin-sensitive tissues. However, distinguishing between them has proven challenging, in part because of a lack of sensitive assays that allow distinct aspects of the intracellular GLUT4 trafficking itinerary to be studied quantitatively in isolation. Further complexity arises based on cell type and differentiation-dependent maturation. Fully differentiated 3T3-L1 adipocytes exhibit strong static retention of insulin-responsive vesicles, whereas preadipocytes and fibroblasts display more dynamic recycling behavior (Fujita et al., 2010; Habtemichael et al., 2018; Harrison et al., 2024; Hatakeyama and Kanzaki, 2010; Muretta and Mastick, 2009; Muretta et al., 2008). Such issues may explain discrepancies among studies supporting "dynamic" versus "static" models.

How does GLUT4 reach the PM?

The intracellular sequestration of GLUT4 is regulated by two mechanisms: static retention within static IRVs, and an intracellular cycle between endosomes – particularly those containing TfR – and a non-endosomal compartment, namely GSCs. A GLUT4 mutant that does not enter this intracellular cycle was found in the PM at higher levels than wild-type GLUT4, but importantly still exhibited substantial insulin-dependent delivery to the PM (Xiong et al., 2010; Xiong and McGraw, 2009). This argues that certain endosomes are intrinsically insulin sensitive, which had been previously suggested based upon insulin-dependent delivery of TfR and GLUT1 to the surface of adipocytes, most likely from recycling endosomal compartments (Calderhead et al., 1990; Tanner and Lienhard, 1987).

Importantly, Govers and colleagues showed that insulin stimulates the release of GLUT4 into the PM recycling system in a graded, insulin dose-dependent manner, a phenomenon they call 'quantal release' (Coster et al., 2004; Govers et al., 2004). They posited that insulin-stimulated delivery of GLUT4 to the PM could not be explained solely by a change in the kinetics of recycling of a fixed GLUT4 pool. Rather, their data is consistent with a model in which GLUT4 is localised to a compartment (IRVs) from which it is released in a quantal fashion in response to insulin (Coster et al., 2004; Govers et al., 2004). GLUT4 then continuously recycles to and from the PM independently of the non-released pool. This is supported by the population level analysis of Muretta et al., described above. Of note, this model and its analysis cannot

distinguish between the released GLUT4 being trafficked directly to the PM, GLUT4 being delivered into endosomes *en route* to the PM, or a combination of both (**Fig. 2**).

Total internal reflection microscopy (TIRFM) is a powerful approach which allows excitation and detection of fluorescent molecules within a few hundred nm of the PM (the 'TIRF zone'). Hence, TIRFM has been useful to capture information on GLUT4 vesicles as they approach the PM, providing details of the numbers of these vesicles, their trajectory or the effect of insulin on their movement (Chen and Saltiel, 2007; Lizunov et al., 2005; Lizunov et al., 2012; Patki et al., 2001). However, it is not always possible to ascribe size information to these structures (see below) and the central question of direct *versus* indirect trafficking cannot be answered using these approaches.

Compelling evidence for what we here will call 'Route 1' – trafficking directly to the PM (**Fig. 2**) – is provided from two key studies. The first considered the trafficking of Rab proteins, well-established regulators of cellular trafficking pathways (Zerial and McBride, 2001), and their overlap with the GLUT4 pathway (Chen et al., 2012). Both Rab10 and Rab14 have been implicated in the delivery of GLUT4 to the PM (Brewer et al., 2016a; Brewer et al., 2016b; Brumfield et al., 2021; Chen and Lippincott-Schwartz, 2013; Chen et al., 2012; Sadacca et al., 2013; Sano et al., 2007; Zaid et al., 2008), and both Rab10- and Rab14-positive vesicles contain the insulin-responsive aminopeptidase IRAP (a marker for GLUT4-containing vesicles). Rab10- and Rab14-positive vesicles translocate to the PM in response to insulin (Chen and Lippincott-Schwartz, 2013; Chen et al., 2012). However, Rab14-vesicles were found to be TfR-positive, whereas Rab10-positive vesicles were not. Ergo, the authors conclude that Rab10-positive vesicles traffic directly to the PM and bypass endosomes, suggesting that this 'Route 1' mechanism could underpin direct insulin-dependent GLUT4 trafficking to the PM from IRVs (Chen and Lippincott-Schwartz, 2013). Rab10 knockdown significantly impaired insulin-stimulated GLUT4 translocation, whereas the effect of Rab14 knockdown was less marked (although still significant). Furthermore, the relative absence of Rab10-positive vesicles within the TIRF zone in the absence of insulin together with a substantial increase in Rab10-positive vesicles in response to insulin suggests that a population of GLUT4 vesicles are sequestered internally away from the cell periphery until such time as insulin is present. These authors posit that Rab 10-positive vesicles contribute majorly to insulin-stimulated GLUT4 translocation by mobilising a 'specialised' pool of GLUT4 vesicles (IRVs) that reach the PM independently of endosomes (Chen et al., 2012). Importantly, they caveat this model with the observation that although insulin utilises both endosomal (Rab14) and endosome-independent (Rab10) mechanisms to mediate GLUT4 delivery to the PM, the latter predominates.

Complementary evidence also comes from Xu et al. (Xu et al., 2011), who used calibrated TIRFM to monitor GLUT4 vesicle fusion at the PM. Their quantitative analysis revealed two distinct GLUT4-vesicle populations present within 3T3-L1 adipocytes: small (~50 nm) vesicles and larger (~150 nm) vesicles. The smaller vesicles were notably absent from undifferentiated cells, suggesting that these represent IRVs. Interestingly, both small and larger GLUT4-vesicles were observed to fuse directly with the PM (Xu et al., 2011). These data suggest that IRVs can follow two principal routes upon stimulation: one subset undergoes direct fusion with the PM ('Route 1'), whereas another subset fuses with TfR-containing endosomes ('Route 2') to enter the recycling network. This dual behavior indicates that IRVs function as integrated insulin-responsive reservoirs, enabling insulin to mobilize GLUT4 through multiple, coexisting trafficking pathways that may be differentially engaged depending on stimulus intensity, duration and metabolic state.

Other studies have also indirectly implicated multiple additional routes to the PM ('Route 2' in **Fig. 2**). These studies include analyses using transferrin-peroxidase conjugates to selectively cross-link and destroy TfR-positive endosomes (termed 'endosomal ablation') (Livingstone et al., 1996), the identification of different soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) requirements for delivering GLUT4 to the surface in response to different stimuli (Martin et al., 1996; Millar et al., 1999; Williams and Pessin, 2008), and comparison of GLUT4 vesicle-associated proteins with known secretory and endosomal markers, such as sortilin and cellugyrin (Kioumourtzoglou et al., 2015; Shi and Kandror, 2005). Of note, endosomal ablation studies found that the fraction of GLUT4 present within recycling endosomes increased upon insulin stimulation (Lampson et al., 2001; Subtil et al., 2000), suggesting that GLUT4 could 're-enter' the recycling endosomal system in response to an insulin signal. However, a characteristic feature of the assays and methods applied to studies of GLUT4 trafficking to the PM thus far has been an inability to directly quantify effects of insulin on specific intracellular steps (including endosome-endosome fusion) and where within the cell these effects may be manifest. As a result, though the consensus in the field is that there are multiple intracellular GLUT4 compartments (the GSC) and that a subset of these are distinct from classical recycling endosomes (the IRV), the complex inter-relationship of these compartments remains difficult to disentangle. The steps that are regulated by insulin remain to be fully explored and, importantly, how these different steps and compartments contribute to the physiological regulation of GLUT4 remains unclear. These and other studies discussed below indicate that PM fusion is not the sole rate-limiting step and underscore the existence of diverse, partially redundant trafficking routes that collectively achieve GLUT4 surface exposure.

Overcoming the barrier to studying compartment-specific intracellular trafficking

Quantum dot (QD) fluorescent nanocrystals are semiconductor particles that offer a powerful tool for live-cell imaging because of their photostability and brightness, enabling fast, sensitive image acquisition and minimizing photobleaching and phototoxicity. Work from the Kanzaki group has allowed high-precision measurements of QD-labelled GLUT4 and TfR to provide the first impetus to reconsider the mechanisms by which intracellular GLUT4 reaches the PM (Fujita et al., 2010; Hatakeyama and Kanzaki, 2011; Hatakeyama and Kanzaki, 2017). Statistical comparison of GLUT4 and TfR behaviour clearly documented a unique regulatory system for GLUT4, identifying putative anchoring mechanisms that statically retain GLUT4 in storage compartments around the perinuclear TGN region. Early work suggested that insulin-responsive 'liberation' from this 'static' GLUT4 anchoring system in adipocytes and skeletal myofibers is a central control point of insulin, as it occurs deep within the cell and likely reflects the most important initial element of insulin-dependent GLUT4 mobilisation (Fujita et al., 2010; Hatakeyama and Kanzaki, 2017).

On one level, this idea is not new: a protein called Tether containing a UBX domain for GLUT4 (TUG) had previously been implicated in the sequestration of GLUT4 (Yu et al., 2007). TUG is thought to either constrain GLUT4 vesicles near the Golgi matrix or function to restrict their budding and fusion (Bogan and Kandror, 2010). Insulin results in cleavage of TUG, which is proposed to release GLUT4 to the PM (Yu et al., 2007). Importantly, over-expression of TUG enhanced the magnitude of insulin-stimulated delivery of GLUT4 to the PM; the number of TUG-GLUT4 complexes that dissociate determines how much GLUT4 is translocated to the PM, providing an intriguing parallel to the 'quantal release' mechanism described above (Belman et al., 2015; Bogan et al., 2012). Importantly, knockdown of TUG was found to increase the fusion of the smaller vesicle population identified by Xu et al. (Xu et al., 2011). Hence, the intracellular trafficking revealed quantitatively by QD analysis not only confirms this idea but extends it by analyzing single GLUT4 molecules and revealing this control mechanism to be deep within the cell, suggesting that it could represent a crucial early regulatory step for GLUT4 translocation. Perhaps most significantly, our (Hatakeyama and Kanzaki) work also identified acute ''heterotypic endosomal fusion'' of very small static GLUT4-containing vesicles with a subset of TfR-containing endosomes as another critical initial process in insulin-responsive GLUT4 translocation, in addition to insulin responsive-liberation, in mouse skeletal myofibers (Hatakeyama and Kanzaki, 2017).

These studies raise crucial questions regarding the relationship between GLUT4 liberation and GLUT4-vesicle fusion as well as their subcellular regional dependence. To address these issues, Hatakeyama and Kanzaki employed a comprehensive imaging-based approach using three distinct live-cell modalities to dissect the spatial and temporal characteristics of insulin-responsive GLUT4 trafficking, focusing on the initial responses upon insulin stimulation (Hatakeyama et al., 2022). Their study integrated QD-based single-molecule tracking, dual-color live imaging and high-resolution fluorescence analysis to elucidate where, when and how GLUT4 molecules are first mobilized and behave within the cell, including the spatiotemporal dynamics of the fusion of GLUT4-vesicles with heterotypic endosomal compartments upon insulin stimulation (Hatakeyama et al., 2022).

As an initial step in this framework, the Kanzaki laboratory employed a dedicated fusion-sensing strategy (Strategy 1, **Fig. 3**) to quantitatively detect heterotypic fusion events between IRVs and TfR-containing vesicles. In this design, myc-GLUT4-mCherry was transiently labeled with an anti-myc antibody covalently crosslinked to BODIPY-conjugated streptavidin (Anti-myc-BODIPY/SA) during brief insulin stimulation, allowing selective tagging of GLUT4 molecules that transiently reached the PM. After insulin withdrawal and return to a quiescent state, the BODIPY-labeled GLUT4 molecules underwent endocytosis and were sequestered back to the static perinuclear pool (IRVs). Subsequently, TfR-positive compartments were labeled by internalization of biotinylated transferrin, allowing differential tagging. Upon insulin restimulation, heterotypic fusion between GLUT4 and TfR compartments occurred, leading to biotin–streptavidin interaction and resulting in BODIPY dequenching, which produced a measurable increase in green fluorescence (**Fig. 3**) (Hatakeyama et al., 2022). This allowed direct and compartment-specific quantification of fusion events throughout the cytoplasm, including in perinuclear and peripheral regions, supporting the view that endomembrane fusion acts as a gateway to GLUT4 mobilization.

To investigate the temporal relationship between heterotypic fusion and GLUT4 molecule liberation (Strategy 2, **Fig. 4**), the authors developed a uniquely integrated dual-labeling strategy that combined the BODIPY/SA-based fusion sensor with QD–based single-particle tracking. In this configuration, each myc-GLUT4 vesicle was simultaneously endowed with two orthogonal signals**:** an anti-myc-BODIPY/SA fusion sensor and sparse labeling with QD655-conjugated anti-myc antibody, enabling nanometer-scale tracking of GLUT4 motility (**Fig. 4**) (Hatakeyama and Kanzaki, 2022; Hatakeyama et al., 2022). This dual-labeling design was technically significant in that it allowed real-time, *in situ* correlation of vesicle fusion and associated GLUT4 dynamics at the single-vesicle level – a capacity not achievable by conventional methods. Low-density QD labeling was particularly critical, as it minimized

optical crowding, thereby enabling accurate single-molecule tracking without compromising the functionality of the fusion sensor. By ensuring precise spatial separation and temporal coordination of the two signals, this strategy enabled direct monitoring of both the heterotypic fusion events and the corresponding behavior of GLUT4 molecules within the same vesicle population (Hatakeyama et al., 2022).

By performing time-resolved dual-channel imaging, the authors were able to correlate the precise onset of GLUT4 molecule motility (as detected by QD displacement) with the timing of fusion-associated BODIPY fluorescence enhancement (Hatakeyama et al., 2022). This approach revealed that the movement of QD-labeled GLUT4 molecules was initiated only after the increase in BODIPY fluorescence, indicating that heterotypic endosomal fusion between IRVs and TfR-containing endosomes is a prerequisite for GLUT4 mobilization toward the endosomal trafficking pathway. These findings provide strong evidence that insulin-responsive heterotypic fusion serves as a physiological gatekeeper, marking the critical transition point at which a GLUT4 molecule is released from a static, storage-sequestered state and integrated into the TfR-containing endosomal network (Hatakeyama et al., 2022).

Complementing these findings, the authors employed a third strategy to examine the presumed post-fusion dynamics of GLUT4 molecules in relation to TfR (**Fig. 5**). In this dual-cargo tracking approach, myc-GLUT4 was labeled with QD655, while transferrin was labeled with QD565, enabling simultaneous single-particle tracking of both GLUT4 and TfR molecules in the same cell (Hatakeyama and Kanzaki, 2013a; Hatakeyama and Kanzaki, 2022). Under basal conditions, QD655-labeled GLUT4 remained largely static and confined to the perinuclear region surrounding the TGN, whereas QD565-labeled TfR exhibited highly mobile, spatially dispersed behavior throughout the cytoplasm. Upon insulin stimulation, GLUT4 molecules underwent a marked transition from a static, perinuclear state to a highly dynamic trafficking mode that closely resembled the motility and spatial distribution of TfR, indicating that GLUT4 had functionally entered the TfR-containing endosomal network (Hatakeyama et al., 2022). This shift was characterized by increased directional movement toward the cell periphery, reflecting a redistribution specific to GLUT4 in response to insulin. TfR vesicles, in contrast, maintained their constitutively high motility and broad cytoplasmic dispersion regardless of the presence of insulin, thereby highlighting GLUT4's selective and insulin-dependent acquisition of this dynamic behavior.

Notably, the heterotypic fusion events occurred preferentially in the perinuclear region enriched with IRVs (Hatakeyama et al., 2022), which are basally retained by Tbc1D-family Rab GTPase-activating proteins (RabGAPs) such as AS160 (Fujita et al., 2010; Hatakeyama and

Kanzaki, 2011) and Tbc1d1 (Hatakeyama and Kanzaki, 2013b; Hatakeyama et al., 2019) through suppression of Rab GTPase activity. This basal retention defines a regulatory checkpoint and fusion in the perinuclear region marks a critical transition toward insulin-responsive GLUT4 trafficking. Importantly, although actin remodelling had long been recognized to have an essential role in insulin-induced GLUT4 translocation (Kanzaki, 2006; Kanzaki and Pessin, 2001; Kanzaki et al., 2001), it has now been directly implicated in the insulin-induced heterotypic fusion of IRVs (Hatakeyama et al., 2022).

It is important that some experimental limitations in the heterotypic fusion studies outlined above are acknowledged. These studies were performed using 3T3-L1 fibroblasts expressing tagged GLUT4, sortilin and AS160 to recapitulate GLUT4 sequestration and insulin responsive trafficking in a simplified cellular context (Hatakeyama et al., 2022). Although adipocyte-specific factors may be absent from these fibroblasts – which could impact trafficking pathways – comparable fusion behavior and regulatory responses have also observed in fully differentiated 3T3L-1 adipocytes as well as in isolated skeletal muscle fibers, supporting the physiological relevance of these findings. Similarly, epitope- and GFP-tagged GLUT4 does not function as a glucose transporter, which might impact its trafficking in ways not presently understood. Such issues exemplify the trade-offs required to capture data from these intrinsically difficult-to-study cell types.

A new regulatory event in the GLUT4 itinerary: fusion-guided GLUT4 entry (FGG4E)

Building on these findings, we propose that the effect of insulin encompasses a fusion-driven step whereby IRVs undergo heterotypic fusion with TfR-positive endosomal compartments, in a manner dependent on both phosphoinositide 3 kinase (PI3K) activity and actin remodeling (Hatakeyama et al., 2022). This enables the selective incorporation of GLUT4 into the recycling endosomal system, facilitating its insulin-dependent delivery to the PM. Importantly, once incorporated, GLUT4 adopts the motile characteristics of TfR, indicating a compartmental transition from a static storage pool to a dynamic endosomal transport stream. This transition reflects not merely vesicle mobilization but a fusion-based molecular integration that grants GLUT4 sustained access to a pre-existing, highly dynamic transport network (**Fig. 6**).

We refer to this mechanism as fusion-guided GLUT4 entry (FGG4E), a process by which insulin-induced membrane fusion initiates a shift in GLUT4 trafficking identity, integrating sequestered vesicles into an actively cycling endosomal pathway (**Fig. 6A**). While insulin stimulation persists, GLUT4 continues to recycle between the PM and endosomes without returning to the original static perinuclear storage pool.

Though it is clear that IRVs can fuse directly with the PM under insulin stimulation (Chen et al., 2012; Xu et al., 2011), we posit that direct delivery likely operates in parallel with or as a subset of the broader FGG4E process. This coexistence of direct and endosome-mediated routes provides flexibility, enabling rapid increases in surface GLUT4 together with sustained endosome recycling. The insulin dose-dependent "quantal release" of GLUT4 and the graded cleavage of the tethering protein TUG further appear to modulate the magnitude of FGG4E activation, linking upstream signaling to the number of vesicles entering this pathway.

The notion that endosomes are a major locus for metabolic homeostasis is not new. Knockdown of Rab5, a small GTPase involved in endosomal sorting, resulted in major metabolic defects including hypoglycemia, hypercholesterolemia and hyperlipidemia (Zeigerer et al., 2015). Other mutations in endosomal genes are associated with metabolic diseases, such as T2DM, hypercholesterolemia and non-alcoholic fatty liver disease (Gilleron and Zeigerer, 2023). Moreover, endosomes are well recognised as signalling hubs for many different hormones and growth factors, including insulin (Gould and Lippincott-Schwartz, 2009). However, do the new observations noted above relating endosomal dynamics to a specific alteration in GLUT4 trafficking provide further evidence to support this notion?

A recent mathematical analysis of GLUT4 recycling provides further food for thought. Romenskaia, Mastick and Coster used the kinetic analysis of GLUT4 recycling, measured using fluorescence activated cell sorting and antibodies against the exofacial HA epitope in HA-GLUT4-GFP, to re-evaluate previous models, this time extending their analysis to how GLUT4 cycling changes both during the transition from no insulin (the basal state) to high insulin, and also at physiologically relevant (and often ill-studied) sub-maximal insulin doses (Romenskaia et al., 2025). They observed that steady-state data fits best to a model that includes a dose-dependent increase in the size of the cycling pool at sub-maximal insulin concentrations (referred to above as quantal release) (Romenskaia et al., 2025). It is tempting to suggest that this increase reflects the sub-endosomal trafficking stimulation described above. Interestingly, these authors also noted that, in addition to increasing the rate constant of GLUT4 exocytosis, insulin also regulates an additional rate constant (i.e. a further trafficking step), suggesting multiple layers of regulation. Some highlights of this compelling analysis include the notion that any sequestered GLUT4 is released into 'internal membrane structures' (perhaps recycling endosomes?) and then recycles to the PM, which accounts for the initially slower rate of appearance of GLUT4 at the PM when insulin is added to 'basal' cells (Romenskaia et al., 2025). This important statement underscores a further issue often ignored in studies of GLUT4

trafficking: to maximise the assay window, we routinely compare cells in the complete absence of insulin with those stimulated with supra-physiological insulin doses. While these approaches have a sound rationale, it is possible that these extreme conditions, which are never encountered *in vivo*, favour one regulatory mechanism over another. By maximising GLUT4 sequestration through incubating cells in the complete absence of insulin, the subsequent addition of very high insulin concentrations could mean that one mechanistic delivery system dominates over another.

A new perspective for physiological regulation?

We suggest that FGG4E offers a rapid and sensitive mechanism to regulate GLUT4 levels at the PM under physiological conditions. In response to sub-maximal (physiological) levels of insulin, Romenskaia et al. note that "there is a rapid release or conversion of a population of GLUT4 vesicles (half time ~3 minutes), then no further release over the course of these experiments despite the continuous presence of insulin" (Romenskaia et al., 2025). This is consistent with previous studies arguing that increasing concentrations of insulin release more vesicles into the cycling pool. Intriguingly, the dose-response characterised by Romenskaia et al. matches that of TUG cleavage (Habtemichael et al., 2018), further emphasising the complex relationships among these distinct mechanisms. Identifying the mechanisms of these actions of insulin represents a future challenge.

Further evidence for an important role for endosomal GLUT4 trafficking has recently been provided. For example, studies of TUG have implicated insulin-stimulated TUG cleavage in glucose uptake in muscles (Bogan et al., 2012; Castorena et al., 2015; Habtemichael et al., 2021) and shown that this insulin-stimulated cleavage is directly correlated with energy expenditure in mice (Bogan, 2022). These studies not only reinforce an important role for TUG but also indicate that FGG4E could have major physiological consequences by acting in tandem with TUG-mediated release of GLUT4 into the endosomal system. Interestingly, studies have argued that the signalling pathways leading to TUG cleavage are subject to both positive and negative regulation (Bogan, 2022), offering the view that the pathways regulating GLUT4 trafficking are 'paradoxical' (Hart and Alon, 2013) – they allow the cell to generate temporal pulses of GLUT4 release and perhaps respond to fold changes in insulin concentration rather than absolute concentrations. By marrying such regulatory controls with the flexibility of multiple routes to the PM, precise control of GLUT4 levels may be achieved in the face of distinct metabolic needs.

A proteomic analysis of GLUT4 compartments using a proximity-ligation based approach in skeletal muscle cells has provided more evidence of the important roles of endosomal GLUT4 trafficking (Ray et al., 2023). Endosomal proteins loom large in this analysis, underscoring the significance of this complex pathway in GLUT4 trafficking, both in response to insulin but also in response to exercise. The latter is further supported by the observation that the overlap between insulin-stimulated GLUT4 with both early and late endosomal markers is greatly increased upon prior exercise (Knudsen et al., 2020). These observations, albeit in a small number of individuals, together with studies describing changes in endosomal protein levels associated with diabetes (Livingstone et al., 2022), strongly argue that endosomal GLUT4 trafficking could be a major physiological control step.

A recent report has examined the breadth of insulin-responsive protein re-localisation in 3T3-L1 adipocytes (Conway et al., 2025). In addition to identifying that many proteins implicated in controlling aspects of endosomal protein trafficking were insulin-responsive, this study identified a potentially important role for endosomal trafficking in insulin action. One of the proteins identified in this screen encodes a Type-III membrane protein called C3ORF18. The subcellular localisation of this protein overlaps with TfR, indicating it is likely localised to recycling endosomes. Strikingly, knockdown of C3ORF18 inhibited insulin-stimulated glucose transport. Interestingly, however, the translocation of both GLUT4 and TfR to the PM was impaired upon C3ORF18 knockdown, offering the intriguing hypothesis that a key focus of insulin is to regulate the insulin sensitivity of endosomal trafficking in general (Conway et al., 2025). The potential clinical relevance of this hypothesis is supported by the observation that *C3ORF18* expression is lower in individuals with higher circulating blood glucose and glycohemoglobin (HbA1c) levels, a marker of aberrant blood glucose control (Conway et al., 2025).

Finally, the idea that GLUT4 uses recycling endosomes to reach the PM is suggested studies which have used knockout or knockdown approaches to probe mechanisms of GLUT4 delivery to the PM (Black et al., 2022; Zhao et al., 2009). Syntaxin-4 is regarded as a key PM target (t)-SNARE for GLUT4 translocation in adipocytes and muscles (Olson et al., 1997; Spurlin et al., 2004; Volchuk et al., 1996). Despite this, ectopic expression of HA-GLUT4-GFP in syntaxin-4-knockout adipocytes showed that although these cells retain significant GLUT4 translocation capacity, the rate constant of GLUT4 exocytosis in synaxtin-4 knockout cells is around 50% slower than that in wild-type cells (Black et al., 2022). It is interesting to speculate that despite the absence of what is regarded as a key agent in GLUT4 delivery to the PM, GLUT4 still finds a way to reach the surface. It is conceivable that syntaxin-4-dependent mechanisms of

translocation dominate under certain experimental conditions, but other mechanisms can step up to be equally as influential in other circumstances.

Collectively, these findings establish the FGG4E mechanism as a key fusion-driven regulatory step that integrates static storage vesicles into the endosomal recycling stream, thereby linking upstream insulin signalling to dynamic GLUT4 trafficking and metabolic responsiveness.

It is important to note that FGG4E may also play an important role in replenishment of IRVs. Upon insulin withdrawal, GLUT4 is retrieved from the endosomal system back to the TGN, allowing repacking into IRVs and restoring static retention (**Fig. 6B**) (Hatakeyama and Kanzaki, 2011; Shi and Kandror, 2005). Complementary to this, Bogan and colleagues proposed that a subset of GLUT4 vesicles may originate from the ER-Golgi-intermediate compartment (ERGIC)-like domain in the perinuclear region, where TUG anchors GLUT4 and IRAP prior to vesicle budding (Orme and Bogan, 2012; Parchure et al., 2025). How these routes relate to FGG4E will likely be the subject of further studies.

Summary

The insights discussed in this Opinion article redefine the early phases of insulin-responsive GLUT4 trafficking, shifting the focus from solely surface translocation to a deeper regulatory gateway: selective entry into the endosomal transport circuit via vesicle fusion. Importantly, this dynamic behavior depends on the prior establishment of a well-organized storage system specific for GLUT4 (Fujita et al., 2010; Hatakeyama and Kanzaki, 2017). In fully differentiated adipocytes and muscle cells, these static retention compartments mature during differentiation, enabling efficient sequestration of GLUT4 molecules under basal conditions and ensuring their readiness for rapid mobilization upon demand (Bryant and Gould, 2020).

Disruption of this static storage system – frequently observed in insulin-resistant states – results in diminished perinuclear retention and impaired GLUT4 responsiveness (Ariga et al., 2008; Fujita et al., 2010; Tsuchiya et al., 2010). This suggests that insulin resistance might, in part, reflect a breakdown of the equilibrium between retention and entry. Some preliminary evidence for this idea comes from studies in which proteins involved in GLUT4 sequestration were found to be decreased in individuals with both obesity and diabetes compared to individuals with only obesity (Livingstone et al., 2022). Hence, it seems reasonable to suggest that maintaining this dynamic balance is crucial for effective glucose uptake. We suggest that such important regulatory mechanisms become more significant under physiological conditions, in which changes in circulating insulin concentrations are far smaller than those traditionally employed in mechanistic studies in the laboratory.

Notably, FGG4E is not limited to adipocytes – similar insulin-stimulated fusion events have been observed in skeletal muscle fibers, supporting the broader relevance of this mechanism across metabolic tissues (Hatakeyama and Kanzaki, 2017). Furthermore, metabolic priming through activation of energy-sensing pathways, such as via 5' AMP-activated protein kinase (AMPK) signalling, has been shown to enhance subsequent insulin responsiveness, likely by promoting the efficiency of GLUT4 incorporation into the endosomal network (Krycer et al., 2020; Krycer et al., 2017; Stockli et al., 2011) (Hatakeyama and Kanzaki, 2013b; Hatakeyama et al., 2019),

Together, these observations reinforce the notion that GLUT4 trafficking is governed by a tightly regulated balance between static sequestration and dynamic circulation and that insulin not only stimulates direct fusion of IRVs with the PM but also delivers GLUT4 to the surface via fusion with endosomes (FGG4E). These processes could be triggered differentially by insulin stimulation and muscle contraction, they might be differentially affected by insulin resistance, and their relative balance and contributions could be dictated by complex regulatory mechanisms, including cellular differentiation status. Understanding the molecular checkpoints that regulate this balance – such as heterotypic fusion and retrograde retrieval – may hold therapeutic potential for restoring insulin sensitivity in metabolic disease.

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Figure legends.

Fig.1. Basal trafficking states of GLUT4: dynamic retention *versus* static sequestration

Comparison of two distinct GLUT4 storage models observed under basal (non-insulin stimulated) conditions. In the static retention model (right), GLUT4-containing vesicles are tightly clustered in peri-nuclear regions and remain essentially immobile, representing a stable sequestration state. In contrast, the dynamic retention model (left) features GLUT4 vesicles that, while dynamically trafficking within a local endosomal system, appear predominantly perinuclear because of steady-state accumulation in this region. These vesicles exhibit limited cycling behavior, consistent with a dynamic retention model, although whether this behavior includes exocytosis to the PM or remains confined to internal membrane systems remains unclear. Both models represent basal states but reflect different degrees of GLUT4 motility and regulatory implications, potentially influenced by cell differentiation status or metabolic history. Insulin and exercise increase delivery of GLUT4 to the PM via distinct signalling mechanisms (see also Fig. 2).

Fig. 2. Two potential routes for insulin- or exercise-stimulated GLUT4 delivery to the PM: direct and/or indirect pathways

Insulin or exercise promotes GLUT4 translocation to the plasma membrane (PM) via two distinct but potentially co-existing routes. In the direct route (Route 1), insulin-responsive GLUT4 storage vesicles (IRVs) translocate and fuse directly with the PM. In the indirect route (Route 2), IRVs first fuse with transferrin receptor (TfR)-positive endosomes, allowing GLUT4 to enter the recycling endosomal system before being delivered to the PM. Both routes are subject to regulation by Akt (induced by insulin) and AMPK (induced by exercise) signaling, which converge on downstream effectors such as the RabGAPs AS160 and Tbc1d1 as well as actin remodeling to facilitate vesicle mobilization and trafficking.

Fig. 3. Selective detection of GLUT4–TfR vesicle fusion via BODIPY dequenching using mCherry as an internal reference.

(A) Illustration of a fluorescence-based fusion sensor strategy utilizing expression of myc-GLUT4-mCherry, anti-myc-BODIPY/SA and biotin-tagged transferrin (Tf) constructs, showing that heterotypic fusion between GLUT4- and TfR-containing compartments leads to dequenching of the BODIPY signal, whereas mCherry fluorescence remains unchanged. Myc-GLUT4-mCherry-positive vesicles are tagged with anti-myc–BODIPY/SA, which remains quenched prior to fusion. TfR-containing endosomes are labeled with Tf-biotin. Upon fusion, biotin–streptavidin interaction enables BODIPY dequenching, resulting in a measurable increase in green fluorescence. The mCherry signal remains constant and serves as an internal reference to correct for fluorescence intensity fluctuations caused by z-axis focal drift or vesicle displacement, thereby allowing selective detection of fusion-dependent BODIPY dequenching. **(B)** Representative images showing the change in fluorescence signal (ΔF) before and after insulin stimulation in 3T3L1 adipocytes expressing myc-GLUT4-mCherry and TfR. ΔF represents the dequenching (enhancement) of BODIPY fluorescence upon heterotypic vesicle fusion. Time-course plots of ΔF and quantification of the area under the curve during the 10-min period after insulin stimulation (ΔF_{0-10}min) are shown. Insulin treatment markedly increased BODIPY dequenching, indicating heterotypic fusion between GLUT4-containing vesicles and TfR-containing endosomes. This response was inhibited by the PI3-kinase inhibitor LY294002. $P < 0.05$. Modified from (Hatakeyama, et al 2022).

Fig. 4. Dual-sensor monitoring of GLUT4 molecule dynamics and heterotypic fusion with TfR endosomes.

(A) Schematic illustration of a live-cell dual-sensor strategy used to detect heterotypic fusion between GLUT4- and TfR-containing compartments, while simultaneously tracking GLUT4 behavior at the single-molecule level. Myc-GLUT4-mCherry vesicles are dual-labeled with anti-myc-conjugated quantum dots (QD) for spatial tracking and with anti-myc–BODIPY/ SA fusion probes, which remain quenched prior to fusion. TfR compartments are preloaded with Tf-biotin. Upon fusion, biotin–streptavidin interaction relieves BODIPY quenching, resulting in a fluorescence increase that signals membrane fusion. Simultaneously, QD-based nanometry provides positional tracking of GLUT4 vesicles. This system

enables precise temporal correlation of vesicle movement and fusion events at the single-vesicle level. **(B)** Representative snapshots of BODIPY fluorescence (green), QD fluorescence (magenta), and the merged images. Modified from (Hatakeyama, et al 2022).

Fig. 5. Transition of GLUT4 from static perinuclear retention to TfR-like motile behavior upon Insulin Stimulation.

(**A**) Schematic representation of 3D trafficking analysis based on GLUT4 distribution and motility. The radial axis (N→PM) represents spatial positioning from the nucleus (N) to the plasma membrane (PM),. The vertical axis (Population, %) indicates population of vesicles exhibiting a given level of trafficking activity at each radial position from the nucleus to the plasma membrane, and the pseudo-color representation illustrates the relative intensity of this activity (low: blue; high: red). This approach captures the transition of GLUT4 from a low-mobility perinuclear state to a more dynamic endosomal population, but it does not provide information on the directionality of movement.

(**B**) Population distribution and trafficking activity of GLUT4- and TfR-containing vesicles under basal and insulin-stimulated conditions. Myc-tagged GLUT4 was labeled with QD655, while transferrin was labeled with QD565, allowing simultaneous single-particle tracking of GLUT4 and TfR in the same cell. Under basal conditions (left), MycGLUT4 is largely perinuclear and low-motility (blue), whereas TfR is more dispersed and motile (red). Upon insulin stimulation (right), GLUT4 adopts a trafficking profile resembling that of TfR, showing increased activity and spatial dispersion toward the cell periphery. These results support the notion that insulin stimulation drives GLUT4 entry into the dynamic endosomal recycling pathway. Modified from (Hatakeyama et al. 2022).

Fig. 6. Fusion-guided GLUT4 entry

(A) Insulin or exercise stimulation initiates a trafficking mechanism termed ‘fusion-guided GLUT4 entry (① FGG4E)’, whereby IRVs undergo heterotypic fusion with TfR-positive endosomal recycling compartments (ERC) near the TGN/ERGIC-like regions. This fusion enables GLUT4 to enter the dynamic recycling endosomal system, where it adopts TfR-like motility and undergoes repeated rounds of trafficking to the PM (② Traffic). Notably, as long as insulin stimulation persists, GLUT4 remains in this dynamic endosomal loop (③ Recycle) and does not return to its original storage pool, suggesting an active mechanism that prevents retrograde retrieval.

(B) Upon cessation of insulin stimulation, GLUT4 is extracted from the recycling pathway through a sortilin- and retromer complex-dependent retrograde trafficking process. This leads to its transient entry into the goligin-97 and glogin0245-positive TGN, where it is momentarily retained and subsequently re-packaged into IRVs. Under basal conditions, GLUT4 is statically sequestered via AS160-dependent mechanisms, remaining in a primed state for the next stimulus. Together, both panels illustrate a reversible cycle of GLUT4 trafficking regulated by stimulus-dependent fusion entry and retrograde resetting, balancing dynamic integration with stable retention. Note: This figure emphasizes the TGN-based retrograde trafficking pathway of GLUT4, which is directly supported by our observations involving sortilin, retromer, and golgin-97/245, and indicates that the GLUT4 vesicle biogenesis system is enhanced upon differentiation.
Nevertheless, we do not exclude the possibility that GLUT4 may also traffic through ERGIC-associated compartments, as suggested by previous studies.

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