# GLUT4 exocytosis

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## Summary

GLUT4 is an insulin-regulated glucose transporter that is responsible for insulin-regulated glucose uptake into fat and muscle cells. In the absence of insulin, GLUT4 is mainly found in intracellular vesicles referred to as GLUT4 storage vesicles (GSVs). Here, we summarise evidence for the existence of these specific vesicles, how they are sequestered inside the cell and how they undergo exocytosis in the presence of insulin. In response to insulin stimulation, GSVs fuse with the plasma membrane in a rapid burst and in the continued presence of insulin GLUT4 molecules are internalised and recycled back to the plasma membrane in vesicles that are distinct from GSVs and probably of endosomal origin. In this Commentary we discuss evidence that this delivery process is tightly regulated and involves numerous molecules. Key components include the actin cytoskeleton, myosin motors, several Rab GTPases, the exocyst, SNARE proteins and SNARE regulators. Each step in this process is carefully orchestrated in a sequential and coupled manner and we are beginning to dissect key nodes within this network that determine vesicle–membrane fusion in response to insulin. This regulatory process clearly involves the Ser/Thr kinase AKT and the exquisite manner in which this single metabolic process is regulated makes it a likely target for lesions that might contribute to metabolic disease.

**Key words:** GLUT4 storage vesicles, GLUT4 trafficking, Exocytosis, Insulin regulation

## Introduction

The mechanisms that govern insulin-stimulated glucose transport in muscle and fat cells have captured the imagination of researchers for decades (see Fig. 1). It all began with Einar Lundsgaard, who observed that insulin increased glucose uptake into muscle of eviscerated cats, whereas the intracellular glucose concentration remained negligible (Lundsgaard, 1939). He concluded that glucose is transported into muscle and that this process, rather than any subsequent step, is rate limiting in the overall glucose clearance process. This was probably the first ‘Eureka’ moment in the field. Since then, many important discoveries have been made (Fig. 1), of which the formulation of the translocation hypothesis (Cushman and Wardzala, 1980; Suzuki and Kono, 1980) and the identification (James et al., 1988) and cloning of the GLUT4 glucose transporter (Birnbaum, 1989; Charron et al., 1989; Fukumoto et al., 1989; James et al., 1989; Kaestner et al., 1989) were possibly the most important, because they put aside other theories that proposed that insulin regulates the intrinsic activity of a transporter.

The translocation of GLUT4 to the plasma membrane following insulin stimulation represents the convergence of two complex systems: signal transduction and vesicular transport. GLUT4 itself has a central role in whole-body glucose homeostasis and defective GLUT4 trafficking might represent one of the earliest defects contributing to insulin resistance in humans (Box 1). Hence, pinpointing the major deterministic nodes for this process could yield important translational benefits for people with metabolic disease. In this Commentary we provide a cell biological perspective of GLUT4 trafficking that focuses on GLUT4 vesicles, their exocytosis and the molecules that control their exocytosis. Several recent reviews have covered other aspects of insulin-regulated glucose transport, including insulin signalling, GLUT4 endocytosis and formation of GLUT4 vesicles. We will not discuss these aspects in detail and direct the reader to these reviews for further information (Bryant and Gould, 2011; Foley et al., 2011; Hoffman and Elmendorf, 2011; Kandror and Pilch, 2011; Rowland et al., 2011). Our review focuses to a large extent on studies carried out in 3T3-L1 adipocytes because this model system has been used extensively for many years and there is an emerging consensus on mechanisms of GLUT4 trafficking in these cells. Important observations about GLUT4 trafficking have been made in other systems such as muscle cells (for a review, see Chiu et al., 2011) and for the most part the overall regulation of this process seems to be conserved between these different systems.

## GLUT4 in a nutshell

Insulin stimulates glucose transport into muscle and adipose tissue 10- to 30-fold with a half time of 2–5 minutes. The major glucose transporter expressed in these tissues is GLUT4. In the absence of insulin, the majority of GLUT4 is stored in small intracellular vesicles [referred to as GLUT4 storage vesicles (GSVs) (Martin et al., 1998) or insulin responsive vesicles (IRVs) (Kupriyanova et al., 2002)]. Following a meal, insulin is secreted by the pancreas and engages its receptor on the surface of myocytes and adipocytes, thereby activating the canonical PI3K–AKT pathway. Activation of this pathway is necessary and sufficient to trigger exocytosis of GSVs to the plasma membrane. This process involves microtubules and actin to navigate GSVs toward the plasma membrane, a tethering apparatus at the plasma membrane (the exocyst complex) to engage and capture GSVs at the cell surface, and fusion machinery (SNARE proteins and SNARE-associated proteins) that merges the GSV lipid bilayer with that of the plasma membrane.

Insulin increases the steady state plasma membrane levels of GLUT4 5- to 30-fold. Three different features of GLUT4 trafficking have been implicated in this redistribution: an increase in the exocytosis rate constant, a decrease in the endocytosis rate constant and an increased amount of GLUT4 in the cell surface recycling

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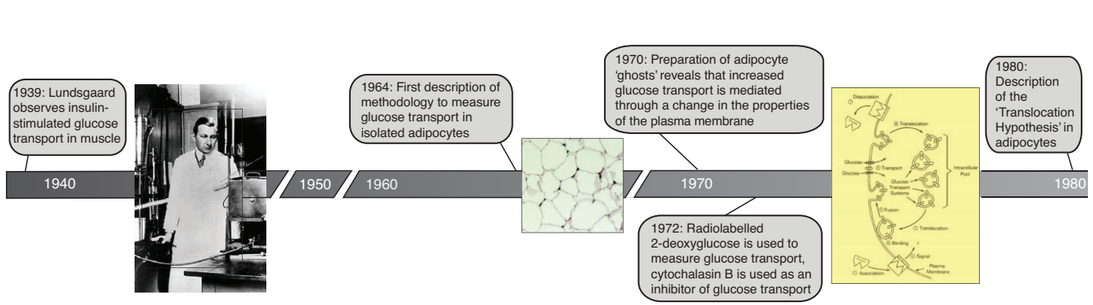



**Fig. 1. A timeline highlighting some of the key events in research on insulin-regulated glucose transport** (Bai et al., 2007; Birnbaum, 1989; Cain et al., 1992; Charron et al., 1989; Cheatham et al., 1996; Cong et al., 1997; Coster et al., 2004; Cushman and Wardzala, 1980; Czech et al., 1993; Dawson et al., 2001; Ebstensen and Plagemann, 1972; Fukumoto et al., 1989; Govers et al., 2004; Hayashi et al., 1998; Huang et al., 2007; James et al., 1988; James et al., 1989; Kaestner et al., 1989; Kanai et al., 1993; Kandror and Pilch, 1994; Karylowski et al., 2004; Katz et al., 1995; Kohn et al., 1998; Kotani et al., 1995; Kurth-Kraczek et al., 1999; Lampson et al., 2001; Lizunov et al., 2005; Lundsgaard, 1939; Martin and Carter, 1970; Martin et al., 1998; Mastick et al., 1994; Merrill et al., 1997; Mueckler et al., 1985; Olson et al., 1997; Quon et al., 1994; Rea et al., 1998; Rodbell, 1964; Sano et al., 2003; Satoh et al., 1993; Shimizu and Shimazu, 1994; Slot et al., 1991a; Slot et al., 1991b; Suzuki and Kono, 1980; Tellam et al., 1997; Volchuk et al., 1996; Wardzala and Jeanrenaud, 1983; Yang et al., 1992)**.** Image of E. Lundsgaard reproduced with permission from BioZoom, published by the Danish Society for Biochemistry and Molecular Biology. Image of 'Translocation Hypothesis' was originally published in *The Journal of Biological Chemistry* (Karnieli et al., 1981) © American Society for Biochemistry and Molecular Biology. Image of GLUT1 is from Mueckler et al. (Mueckler et al., 1985). Reprinted with permission from The American Association for the Advancement of Science.

pool. The relative contribution of each of these parameters to GLUT4 translocation can vary between different cell types and different experimental conditions (Antonescu et al., 2008; Coster et al., 2004; Fazakerley et al., 2010; Govers et al., 2004; Karylowski et al., 2004; Martin et al., 2006; Muretta et al., 2008).

## GSV theories – fact or fiction?

### Theory 1: GLUT4 resides in specialised vesicles, or GSVs, that undergo insulin-dependent translocation to the plasma membrane

A range of approaches have been used to show that GLUT4 resides in small, 50–70 nm GSVs as well as larger structures that are likely to be derived from, or subcompartments of, the trans-Golgi network (TGN) and endosomes. However, as summarised in Box 2, distinguishing between these different GLUT4 compartments using fluorescence microscopy has been difficult, if not impossible.

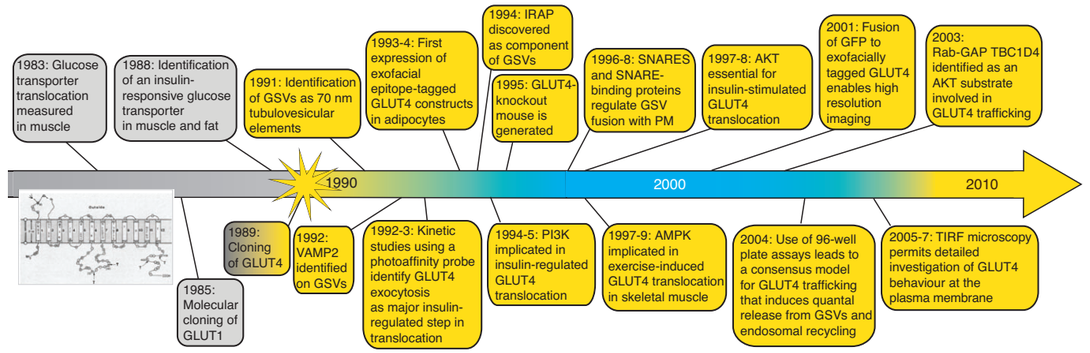
Biochemical approaches to isolate GSVs led to the identification of the insulin-regulated amino peptidase (IRAP) as a GSV component (Kandror et al., 1994; Keller et al., 1995). This protein has a similar trafficking pattern to GLUT4 and the two proteins are highly colocalised (Martin et al., 1997; Ross et al., 1996). Although the function of IRAP translocation in response to insulin is not known, the concept that GLUT4 shares its cellular location with other proteins supports the notion of a specialised GLUT4 compartment. Other proteins, such as the v-SNARE VAMP2, low density lipoprotein receptor-related protein 1 (LRP1) and sortilin were also identified on GSVs, but these proteins do not share the same degree of co-occupation as GLUT4 and IRAP (Cain et al., 1992; Jedrychowski et al., 2010; Lin et al., 1997). Whereas it has been possible to segregate the larger GLUT4-containing compartments, such as TGN and early endosomes, from smaller GLUT4 vesicles using biochemical approaches, it has been more difficult to determine the homogeneity of the small vesicles (Kandror and Pilch, 2011). It is highly likely that the preparation of small vesicles represents a heterogeneous population that comprises GSVs as well as many transport vesicles of <150 nm in diameter. More sophisticated separation techniques have described subpopulations of small, highly insulin-responsive GLUT4 vesicles and a separate population that is probably derived from endosomes and/or the TGN and exhibits little insulin responsiveness (Hashiramoto and James, 2000; Kupriyanova et al., 2002). The insulin-responsive vesicle population is also enriched in IRAP and VAMP2, so these vesicles probably correspond to bona fide GSVs (Kupriyanova and Kandror, 2000; Livingstone et al., 1996).

Immuno-electron microscopy (immuno-EM) has been the most powerful tool for resolving different GLUT4 compartments. This approach was pivotal in showing that, although GLUT4 is found in endosomes and the TGN in adipocytes and muscle cells, the majority of the protein is found in small tubulovesicular elements that are scattered throughout the cytoplasm. These latter structures are the principal donors for insulin-dependent GLUT4 translocation to the plasma membrane (Slot et al., 1991a; Slot et al., 1991b) and subsequent studies identified them as GLUT4-enriched vesicles with a diameter of 50-70 nm (Martin et al., 2000). Whole-mount immuno-EM studies of cells or isolated vesicles showed that there was considerable heterogeneity in the amount of GLUT4 per vesicle, which can range from 1 to 25 molecules per vesicle (Martin et al., 2000; Ploug et al., 1998; Ramm et al., 2000) (Fig. 2). Double labelling of different proteins revealed that GLUT4-laden vesicles are also rich in IRAP and VAMP2, but do not contain endosomal or TGN proteins, and vice versa.

In summary, biochemical data and EM revealed that GSVs do exist. They provide storage and exocytic functions for GLUT4 and a handful of other proteins, such as IRAP, thereby enabling transient regulation of nutrient intake into muscle and fat cells on demand.

### Theory 2: Intracellular GLUT4 sequestration is mediated by retention or repulsion

Kinetic analyses of GLUT4 recycling by tracking antibody-tagged GLUT4 molecules in living adipocytes have shown that, in the absence of insulin, GLUT4 recycles slowly between GSVs and the plasma membrane compared with GLUT4 translocation in the




presence of insulin or with other recycling membrane proteins such as the transferrin receptor (TFR) (Blot and McGraw, 2008; Govers et al., 2004; Habtemichael et al., 2011; Karylowski et al., 2004; Muretta et al., 2008). Whereas there are some quantitative discrepancies in the degree of plasma membrane recycling in the absence of insulin between various studies, presumably owing to differences in the techniques used, a consensus is emerging (see below). By contrast, trying to explain these differences with terms such as 'dynamic' versus 'static' retention is more problematic (Dugani and Klip, 2005; Martin et al., 2006; Muretta et al., 2008). The term 'dynamic' was originally used to describe high basal recycling, whereas 'static' referred to low basal recycling. However, the term 'static' precludes a model in which GSVs constantly (dynamically) move toward the plasma membrane with limited access to the fusion machinery in the absence of insulin. Thus, we suggest two new terms to differentiate between these different models of basal intracellular sequestration of GLUT4: retention, to describe the GSVs being physically prevented from moving towards the plasma membrane and repulsion, to describe the situation in which GSVs move close to the plasma membrane but are unable to fuse with it.

Retention of GSVs could involve a mechanism that anchors the vesicles in the interior of the cell or restricts their access to the (cytoskeletal) tracks that shuttle them to the plasma membrane. In adipocytes, static pools of GLUT4 that become more mobile with insulin have been reported using live cell fluorescence microscopy (Fujita et al., 2010). However, in this study no distinction was made between GSVs and other GLUT4-containing compartments such as endosomes and the TGN. Taking into account the resolution limits of light microscopy (Box 2), these studies might selectively visualise the larger GLUT4-positive structures. It is possibly not surprising that these structures also undergo insulin-dependent changes, because insulin regulates numerous vesicle transport pathways in adipocytes (Tanner and Lienhard, 1987) and has a significant effect on endosomal morphology (Slot et al., 1991b). Therefore, the initially static structures might represent GLUT4 in TGN and/or endosomes, possibly en route to GSVs.

The retention model implies that GSVs must bind to other molecules that impede their mobility. The proteins tether containing UBX domain for GLUT4 (TUG or ASPC1) and the Rab GTPase-activating protein (GAP) TBC1D4 (also known as AS160, Box 3) are potential candidates because they interact directly with GLUT4 or IRAP (Bogan et al., 2003; Larance et al., 2005; Peck et al., 2006; Yu et al., 2007). Disruption of TUG increases glucose transport and GLUT4 translocation under basal conditions and inhibits insulin action (Yu et al., 2007). However, whereas TUG probably has a role in GLUT4 trafficking, it is unclear whether it acts as an intracellular retention anchor. Rather, TUG might regulate GLUT4 endosomal sorting (Fig. 3). Consistent with this, in TUG-depleted adipocytes, GLUT4 relocalises to large structures that resemble endosomes (Yu et al., 2007).

TBC1D4 has GAP activity towards a number of Rab GTPases that are found on GLUT4 vesicles (Miinea et al., 2005). The direct interaction of TBC1D4 with GSV cargo is thought to inactivate a Rab, which renders GSVs fusion-incompetent (Box 3). Rab proteins have roles in vesicle budding, attachment of vesicles to microtubules and in vesicle docking with their target membrane (Zerial and McBride, 2001). Thus, if TBC1D4 controls GTP loading of a Rab that facilitates binding of GSVs to microtubules, this could confer GSV retention. Intriguingly, Rab4 is found on GLUT4 vesicles (Aledo et al., 1995) and it binds to the microtubule motor protein kinesin family member 3 (KIF3) in an insulin-dependent manner (Imamura et al., 2003; Semiz et al., 2003), but no link between Rab4 and TBC1D4 has been shown. It would thus be informative to determine whether other Rab proteins could be involved in mediating retention.

In contrast to retention, the repulsion model suggests that GSVs constantly access the plasma membrane, irrespective of the presence of insulin. Without insulin, GSVs are repelled from the plasma membrane, whereas insulin overcomes this and leads to fusion of the GSVs with the plasma membrane. Is there evidence for GSV repulsion at the plasma membrane? The application of total internal reflection fluorescence (TIRF) microscopy to GLUT4 trafficking has shed light on this question. This technique, when applied to adipocytes expressing GFP-tagged GLUT4, enables visualisation of GLUT4-containing structures within 150 nm of the plasma membrane, which is referred to as the TIRF zone. Several groups have described numerous GLUT4-positive compartments close to the plasma membrane, even in the absence of insulin (Bai et al., 2007; Huang et al., 2007; Lizunov et al., 2005). In some cases, they become stationary beneath the membrane, a step that is thought to be analogous to docking, but in the absence of insulin, these compartments rarely fuse with the plasma membrane. This favours the repulsion model, where GLUT4 vesicles are constantly cycling in and out of the TIRF zone, but in the absence of insulin they have a low probability of fusion. Intriguingly, there is a small pool of TBC1D4 at the plasma membrane that is highly phosphorylated in the presence of insulin (Ng et al., 2010) and this

### Box 1. GLUT4 in whole-body glucose homeostasis and disease

**Lessons from recombinant mouse studies**

GLUT4 expression is highest in adipose tissue and skeletal muscle, but GLUT4 is also found in other organs such as brain, kidney and intestine (Brosius et al., 1992; Rayner et al., 1994; Stöckli and James, 2009) and its possible role as a glucose sensor in these and other organs is worthy of future investigation.

A global reduction in GLUT4 (as occurs in mice heterozygous for GLUT4) results in diabetes (Stenbit et al., 1997), whereas selective disruption of GLUT4 expression in muscle or adipose tissue induces global insulin resistance (Abel et al., 2001; Zisman et al., 2000). The mechanism for intra-tissue communication is unclear, although it has been proposed that insulin signalling in other tissues might be attenuated by an adipokine in adipose-specific GLUT4-knockout mice (Yang et al., 2005). Despite this, it is of interest that genetic ablation of GLUT4 in adipose tissue and/or skeletal muscle does not lead to diabetes (Abel et al., 2001; Kotani et al., 2004; Zisman et al., 2000).

GLUT4 overexpression in adipose tissue and/or muscle enhances glycaemic control in mice (Kotani et al., 2004; Tsao et al., 1996). These studies have identified glucose transport as the rate-limiting step in glucose utilisation because adipose and muscle tissue with enhanced GLUT4 expression exhibit higher levels of triglyceride and glycogen, respectively (Shepherd et al., 1993; Tsao et al., 1996).

**GLUT4 in disease**

In humans, no mutations in *GLUT4* that are linked to metabolic disease have been identified. Furthermore, polymorphisms in *GLUT4* are rare and equally prevalent in both normoglycaemic individuals and type 2 diabetics (Buse et al., 1992). In patients with type 2 diabetes, GLUT4 mRNA and protein levels are reduced in the adipose tissue but not in muscle (Garvey et al., 1992; Garvey et al., 1991; Pedersen et al., 1990; Sinha et al., 1991). However, on the basis of the phenotype of adipose-specific GLUT4-knockout mice, reduced GLUT4 levels in adipose tissue might contribute to the development of whole-body insulin resistance.

Insulin-stimulated glucose transport into skeletal muscle is a major defect in human diabetes (Rothman et al., 1992) but this cannot be explained by changes in GLUT4 levels. The exact point of the defect is currently not known but there is no evidence to refute a major defect in the machinery that regulates GLUT4 translocation as a major contributor to muscle insulin resistance. This represents one of the most important questions in the field, which will hopefully be addressed by future research.

could denote a function of TBC1D4 in disarming a Rab that regulates docking and fusion of GSVs at the plasma membrane.

In conclusion, although there is evidence for retention and repulsion of GLUT4 vesicles, the contribution of each to GLUT4 trafficking is not clear, principally because higher-resolution approaches are required to distinguish between endosomes and GSVs.

### Theory 3: GSVs fuse directly with the plasma membrane

TIRF provides an ideal tool to examine this theory; however, early studies using GFP-tagged GLUT4 failed to detect many cell surface fusion events, probably owing to high background fluorescence that resulted from the labelling of numerous compartments within the TIRF zone. In addition, it is difficult to distinguish between the arrival of GSVs in the TIRF zone, their docking to the plasma membrane and actual fusion using GLUT4–GFP (Lopez et al., 2009). The use of pH-sensitive probes such as pHluorin that can be attached to proteins located on GSVs such as IRAP or VAMP2 overcame some of these problems (Jiang et al., 2008; Xu et al., 2011). This probe comprises a pH-sensitive variant of GFP that is quenched at low pH but fluoresces at neutral pH (Miesenbock et al., 1998). The number of fusion events observed with these probes was substantially higher than with GLUT4–GFP, in part because of the marked reduction in background fluorescence. Moreover, fusion events are accompanied by a bright, rapid burst of fluorescence as the probe encounters the pH-neutral external environment. IRAP-pHluorin was used to analyse fusion of GLUT4-containing vesicles (Jiang et al., 2008). Because IRAP and pHluorin-labelled IRAP are highly colocalised with GLUT4 (Jiang et al., 2008; Martin et al., 1997), it seems reasonable to assume that this pH-sensitive reporter mirrors GLUT4 behaviour. Using IRAP–pHluorin, a 20-fold increase in fluorescence at the plasma membrane following fusion and a >40-fold increase in fusion rate could be detected compared with GLUT4–GFP (Jiang et al., 2008). Although many more fusion events were detected with this probe, the precise nature of the fusing vesicles was not established. If indeed these structures do represent GSVs this represents an important step forward, overcoming many problems encountered with fluorescence microscopy (see Box 2).

More recent studies have observed a rapid burst of fusion of IRAP–pHluorin vesicles in adipocytes that peaked at 3–4 minutes after insulin stimulation and then decayed back to a level slightly above baseline (Stenkula et al., 2010). This might indicate rapid fusion of GSVs, but this was not proven. In a separate study also in adipocytes, it was shown that initial VAMP2–pHluorin fusion events were mediated through small carriers (56 nm), whereas later events were mediated by larger carriers (150 nm) (Xu et al., 2011). However, a numerical simulation of fluorescence intensity in the TIRF zone was used to obtain these measurements and so further studies involving direct measurement of particle size using high-resolution methods are required to validate these observations.

In conclusion, there is evidence that GSVs directly fuse with the plasma membrane, but this occurs as a transient burst and so further effort is needed to confirm whether GLUT4 continues to recycle back to the plasma membrane in GSVs or in endosomes in the continued presence of insulin.

## A GLUT4 trafficking model

It is pertinent at this point to consider how GLUT4 shuttles between the plasma membrane and intracellular GLUT4 compartments to provide a clear framework for the molecular regulation of this process. Pulse-chase cell-surface labelling experiments combined with either endosomal ablation or re-sialylation as a measure of retrograde transport to the TGN, indicate that GLUT4 rapidly enters endosomes following insulin stimulation (within 5 minutes). Later, it moves to the TGN, as demarcated by colocalisation with syntaxin-6 and syntaxin-16 and re-sialylation of IRAP (Blot and McGraw, 2008; Shewan et al., 2003). These observations fit with earlier EM localisation studies (Slot et al., 1991a; Slot et al., 1991b). In the absence of insulin, GLUT4 is detected in the TGN, in small vesicles and tubules that are presumably GSVs, whereas only low labelling of endosomes is observed under these conditions (Slot et al., 1991a; Slot et al., 1991b). Following insulin stimulation, there is a considerable reduction in labelling of the small vesicles and tubules concomitant with increased labelling of endosomes.

These observations lead to a model (Fig. 3) whereby, in the absence of insulin, GLUT4 is effectively sequestered from the

plasma membrane recycling pathway through sorting into GSVs and retrograde transport from endosomes to the TGN (Blot and McGraw, 2008; Coster et al., 2004; Govers et al., 2004; Muretta et al., 2008; Shewan et al., 2003). The TGN cycle could either be a back-up mechanism to sequester GLUT4 that is awaiting entry into GSVs or it might serve a biosynthetic role in the birth or re-birth of GSVs, or both. In response to insulin, GSVs are discharged and fuse directly with the plasma membrane. The recent TIRF data (Stenkula et al., 2010) and the kinetic analysis of GLUT4 recycling data (Coster et al., 2004; Muretta et al., 2008), support the notion that GSVs are not regenerated in the continuous presence of insulin. Instead GSVs are released in a burst, and once discharged to the plasma membrane, GLUT4 undergoes repeated rounds of endocytosis and recycling (Fig. 3). There are several potential endosomal recycling routes: a direct, rapid pathway from early endosomes, a slower pathway involving recycling endosomes and possibly a third involving the TGN (Sheff et al., 1999; Stoorvogel et al., 1988). It has not yet been resolved which of these takes GLUT4 back to the plasma membrane in the continuous presence of insulin. Whereas earlier studies concluded that this pathway was overlapping with the TFR recycling pathway (Livingstone et al., 1996; Zeigerer et al., 2004), recent studies have challenged this (Habtemichael et al., 2011). Intriguingly, GLUT4 is internalised through both clathrin-dependent endocytosis and clathrin-independent and cholesterol-dependent endocytosis (Blot and McGraw, 2006). Conversely, the TFR is internalised only through the clathrin-dependent pathway suggesting that the GLUT4-specific clathrin-independent pathway contributes to the sorting of TFR and GLUT4 into separate intracellular compartments (Blot and McGraw, 2006). Following insulin withdrawal, GLUT4 traffics through the TGN (Shewan et al., 2003), which leads to re-formation of GSVs.

In summary, it is clear that multiple carriers transport GLUT4 to the plasma membrane in adipocytes. This is important because most of our molecular understanding of GLUT4 trafficking is based on studies that do not distinguish between different types of carriers (see Box 2). Hence it is unclear whether many of the molecules that are thought to regulate GLUT4 trafficking regulate fusion of GSVs or other GLUT4-containing compartments with the plasma membrane. The ability of GLUT4 to move to the plasma membrane in different carriers might explain the redundancy in this system that has been observed upon deletion of certain key components such as VAMP2 or Munc18c (Kanda et al., 2005; Zhao et al., 2009).

## Molecular mechanisms of vesicle fusion

In this section, we focus on the molecular regulation of GLUT4 trafficking near or at the plasma membrane; for a review of intracellular GLUT4 sorting, we refer the reader to Kandror and Pilch (Kandror and Pilch, 2011). As mentioned in Box 2, most studies do not distinguish between GSVs and other GLUT4-containing vesicles that fuse with the plasma membrane, and so, for the purpose of accuracy, we confine this discussion to all GLUT4-containing vesicles, referred to here as GCVs. As is the case for most vesicle transport processes, there are at least three kinds of close encounters of GCVs with the plasma membrane before fusion: the approach, tethering and docking (Fig. 4). Here, we consider the molecular regulation of these steps with regard to GLUT4 trafficking in adipocytes.

### Box 2. GSVs cannot be easily seen

In the cell, GLUT4 exists in small, insulin-responsive vesicles that are often referred to as GSVs, as well as larger structures that are derived from endosomes and the TGN. A further complication in the visualisation of GSVs is that GLUT4 is also located in small non-insulin-responsive vesicles that probably correspond to transport vesicles that shuttle between endosomes and the TGN or other organelles (Kandror and Pilch, 2011). Distinguishing between all of these compartments is extremely difficult, yet many investigators often refer to all GLUT4-containing vesicles as GSVs. This is particularly problematic for morphological studies as GSVs cannot be (easily) identified by light microscopy, because they are diffraction limited and cannot be reliably resolved.

Although in isolation a single 70 nm GSV can be readily resolved by fluorescence microscopy, in the context of the cellular environment two major problems arise that seriously limit their identification. A single GSV 70 nm in diameter will be indistinguishable from larger structures with diameters up to 250 nm. In addition, the ability to detect 70 nm GSVs will be limited by the presence of high background fluorescence derived from larger, neighbouring GLUT4-positive structures. Hence, most of the GLUT4 labelling that is typically observed using all traditional fluorescence microscopy techniques, including confocal, epifluorescence and total internal reflection fluorescence (TIRF), probably corresponds to larger endosomal or TGN-related structures (Fig. 2). A further problem with immunofluorescence studies performed on fixed cells is that extraction methods that facilitate antibody accessibility often result in differential loss of certain compartments, and small vesicles are likely to be particularly susceptible to this artefact. A similar problem also applies to biochemical approaches, where in most cases the necessary attempts are not made to distinguish between GSVs and other GLUT4 vesicles, yet investigators often refer to GSVs rather than non-distinct GLUT4 containing vesicles (GCVs). Throughout this review we have tried to use nomenclature that appropriately distinguishes between these different compartments.

### Approach

In many systems, the cytoskeleton provides a route for vesicles to approach the plasma membrane. In adipocytes, these tracks are unlikely to be provided by actin, because adipocytes do not possess obvious stress fibres and instead have an abundance of cortical actin just beneath the plasma membrane (Fig. 2G,H). Hence, microtubules, which are found in a complex network throughout the adipocyte, probably deliver GLUT4 vesicles to the cellular cortex (Semiz et al., 2003), and insulin-regulated kinesin motors have been described (Imamura et al., 2003; Semiz et al., 2003). Notably, in cardiac muscle (Slot et al., 1991a) and rat primary adipocytes (Malide et al., 2000) GLUT4 is concentrated in small vesicles that are often found at distances of <100–200 nm from a surface membrane, in which case long-range microtubule-dependent movement might not have a main role in the translocation of these GCVs, but rather in the more persistent delivery of GLUT4 to the plasma membrane, which might be mediated by endosomes.

### Tethering

The formation of ternary SNARE complexes that mediate fusion occurs when membranes are separated by 4–8 nm (Li et al., 2007). However, the first meaningful interaction between exocytic vesicles and the plasma membrane probably occurs at greater distances and

such tethers are mediated by large multi-subunit complexes or extended rod-like molecules (Brown and Pfeffer, 2010). Many tethers have been identified, each operating at distinct transport steps. Here we describe the possible role of three tethers in GLUT4 trafficking – the exocyst, actin and TBC1D4 (Fig. 4).

Disruption of exocyst function affects many transport steps, including vesicle transport to the bud neck in yeast (TerBush et al., 1996), basolateral TFR recycling, apical recycling of the IgA receptor and transcytosis in MDCK cells (Oztan et al., 2007) as well as GLUT4 trafficking in adipocytes (Inoue et al., 2003). The exocyst is comprised of eight subunits and interacts with many molecules: phospholipids, actin, actin nucleators (Arp2/3, IQGAP1), atypical protein kinase C and JNK, small molecular GTPases (RAL, CDC42, Rho, Rab8, Rab10, Rab11, TC10, ARF6) as well as SNAREs and SNARE-associated proteins such as snapin (Bao et al., 2008; Das and Guo, 2011; Hertzog and Chavrier, 2011). A low-resolution EM structure reveals that the exocyst forms a 'Y'-shaped structure (Hsu et al., 1998). It has been proposed that each tip of the 'Y' interacts with the plasma membrane and the transport vesicle through different small GTPases (Munson and Novick, 2006). The observation that Rab10 and Rab11 are both found on GLUT4 vesicles and interact with the exocyst suggests that these proteins constitute part of the tethering machinery for GLUT4-containing vesicles at the plasma membrane (Jedrychowski et al., 2010; Larance et al., 2005).

The majority of actin in the adipocyte is cortical (Fig. 2G,H), and its depolymerisation or stabilisation inhibits insulin-dependent GLUT4 trafficking (Lopez et al., 2009; Omata et al., 2000; Tong et al., 2001; Tsakiridis et al., 1994). Insulin causes actin rearrangements and changes in cell shape in adipocytes and muscle cells (Martin et al., 1996). Although the structure of cortical actin in the adipocyte has not been examined in detail, it probably extends far enough into the cytoplasm (Fig. 2G,H) to make it an attractive candidate for a longer-range tether compared with the exocyst, which is estimated to only bridge distances of ~15 nm (Munson and Novick, 2006). Myosin motors, including MYO5 and MYO1C, have been identified on GLUT4 vesicles and at the plasma membrane, making them candidate molecules that link GCVs, actin and the plasma membrane (Bose et al., 2002; Yip et al., 2008; Yoshizaki et al., 2007). Interestingly, MYO5 binds to Rab8, Rab10 and Rab11, whereas MYO1C binds to RAL (Chen et al., 2007; Roland et al., 2011). These molecules have all been implicated in GLUT4 trafficking and they all bind to the exocyst complex (Babbey et al., 2010; Das and Guo, 2011; Moskalenko et al., 2002). Collectively, there is an intriguing relationship between GLUT4 vesicles, cortical actin, the exocyst and SNARE proteins at the plasma membrane in the adipocyte, although establishing the hierarchical relationship between these

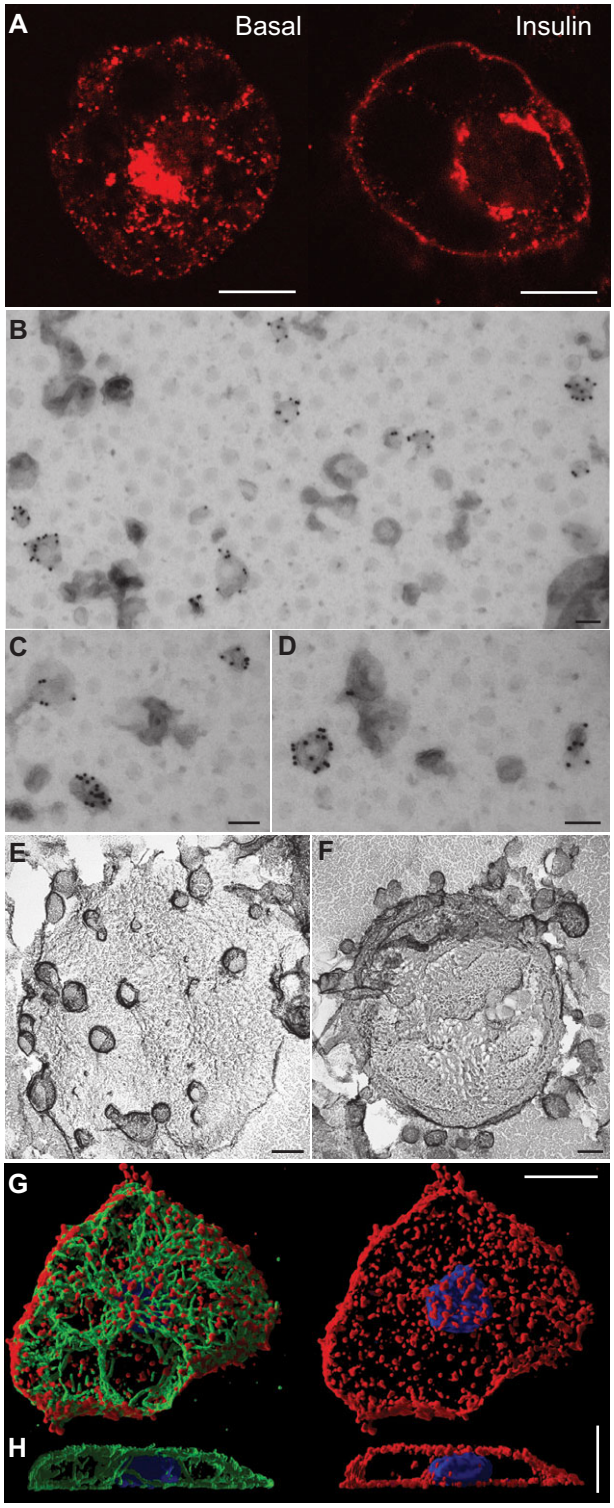



**Fig. 2. Imaging GLUT4 and the cytoskeleton in adipocytes.** (**A**) Immunofluorescence image of untreated (left) or insulin-stimulated (right) 3T3-L1 adipocytes that were immunolabelled for endogenous GLUT4. (**B–D**) Immuno-EM of GLUT4 in vesicles that were prepared from non-stimulated rat adipocytes. After adsorption to EM grids the vesicles were labelled with an antibody against GLUT4, followed by protein-A–gold (10 nm). The size of GLUT4 vesicles in B ranges from 63 to 135 nm. Images were taken by Sally Martin [see Martin et al. (Martin et al., 1997) for methodological details]. (**E**,**F**) Deep-etch images of GLUT4 vesicles immunopurified using *Staphylococcus aureus* cells coated with an anti-GLUT4 antibody then incubated with an adipocyte microsomal fraction. Non-GLUT4-containing vesicles were washed away. Consistent with B–D, the majority of vesicles are small (~50–70 nm) but a population of larger vesicles was also detected. Images were taken by John Heuser using a published method (Rodnick et al., 1992). (**G**,**H**) 3D shadow renderings of 120 confocal *z*-sections (Imaris, Bitplane) of an untreated 3T3-L1 adipocyte. Tubulin (stained with an anti-tubulin antibody) is shown in green, F-actin (stained with TRITC–phalloidin) is shown in red and the nucleus (labelled with DAPI) is shown in blue. Viewed from the top (G) or from the side (H). Scale bars: 10 μm (A,G,H) and 100 nm (B–F).

molecules will be challenging (Fig. 4). Does actin promote exocyst assembly or vice versa? Do actin cables provide a slide for GCVs to navigate toward the plasma membrane, what is the motor for this, and are the appropriate SNAREs found at the bottom of the slide? Further studies will be required to answer these and other questions that still remain with regards to GCV tethering at the plasma membrane.

TBC1D4 (also refer to Box 3) is a 160 kDa protein that possesses tether-like features through the formation of homo-oligomers (Dash et al., 2009) and binds to GLUT4 vesicles (Larance et al., 2005) as well as the plasma membrane (Ng et al., 2010). The molecular details of these interactions are not yet known. In view of its functional link to multiple Rab proteins (Miinea et al., 2005), three of which interact with the exocyst and MYO5 (Babbey et al., 2010; Das and Guo, 2011; Roland et al., 2011), it seems possible that TBC1D4 has a crucial role in bridging the interaction between Rab proteins on the GCVs, actin and the exocyst.

### Docking and fusion

The minimal and universal machinery for membrane fusion in eukaryotes is comprised of a SNARE ternary complex and a Sec1/Munc18-like (SM) protein (Fig. 4). But these proteins are thought to be constitutively active, at least in vitro (Sudhof and Rothman, 2009). Therefore, additional regulatory molecules are required to either silence or activate SNAREs to provide a checkpoint for insulin-regulated trafficking (see the 'repulsion' model above).

Syntaxin-4 and SNAP23 represent the t-SNAREs, VAMP2 the v-SNARE and Munc18c the SM protein involved in GCV fusion with the plasma membrane (Bryant et al., 2002). Whereas SNAREs impart specificity, a degree of promiscuity among these proteins has been described. Syntaxin-4 and SNAP23 can form ternary complexes with VAMP2, VAMP3 and VAMP8 (Wang et al., 2010), and VAMP3 or VAMP8 can substitute for VAMP2 with no apparent perturbation in insulin regulation of GLUT4 trafficking (Zhao et al., 2009). The SNARE–SM complex is considered to have an essential role in bringing two separate bilayers, in this case GCVs and the plasma membrane, together. Because the v-SNARE in the GCV forms a stable complex with the t-SNAREs in the plasma membrane in a zipper-like process (Melia et al., 2002), the closing of the zipper is thought to bring the two membranes closer together to overcome energy barriers that would otherwise prevent such a merger. Munc18c aids in building this complex effectively, as do other molecules that can assist or prevent the zippering process (Hu et al., 2007; Latham et al., 2006; Sudhof and Rothman, 2009). In the case of GLUT4 trafficking, one such molecule could be tomosyn, which possesses a VAMP2-like domain that forms a complex with syntaxin-4 and SNAP23 (Widberg et al., 2003).

The precise role of Munc18c in SNARE assembly is unclear, but its deletion has a modest effect on GLUT4 trafficking in adipocytes (Kanda et al., 2005). One model is that syntaxin proteins exist in open (active) or closed (inactive) conformations and Munc18 facilitates the 'closed' conformation. This hypothesis is based on an X-ray crystal structure of the neuronal proteins, syntaxin-1a and Munc18-1 (Misura et al., 2000), which showed Munc18-1 cradling syntaxin-1a in a closed conformation. However, there is little evidence to suggest that the closed binding mode of syntaxin is a universal feature, but instead represents a peculiarity of the neuronal isoform (Gerber et al., 2008). In fact, a crystal structure involving an N-terminal peptide of syntaxin-4 and Munc18c (Hu et al., 2007), is consistent with a model in which Munc18c binds to open syntaxin-4. Munc18c also binds to the ternary SNARE complex (Latham et al., 2006), which would support more of a positive regulatory role for Munc18c. However, the role of Munc18c in GLUT4 trafficking remains controversial, because it has been shown to be inhibitory when overexpressed in

### Box 3. GAPs and Rabs

The Rab-GAP TBC1D4 has been implicated in insulin-stimulated GLUT4 translocation (Kane et al., 2002; Larance et al., 2005). TBC1D4 phosphorylation in response to insulin regulates its interaction with 14-3-3 (Ramm et al., 2006; Sano et al., 2003). Overexpression of a mutant protein that cannot be phosphorylated (TBC1D4-4P) in adipocytes inhibits insulin-stimulated GLUT4 translocation. Abolition of the GAP activity or addition of a constitutive 14-3-3 binding site to TBC1D4-4P reverses this inhibition, which indicates that inactivation of the TBC1D4 GAP activity, possibly through binding14-3-3, is required for GLUT4 translocation. This suggests that TBC1D4 inactivates a Rab on GLUT4 vesicles. Insulin-stimulated phosphorylation and 14-3-3 binding inhibit the TBC1D4 GAP activity, which then leads to Rab activation and GLUT4 translocation. TBC1D4-4P inhibits movement of GLUT4 vesicles towards the plasma membrane and docking at this site, but has no effect on fusion (Jiang et al., 2008; Xiong et al., 2010). We suggest two models to explain this: (1) TBC1D4 regulates movement of vesicles to the cell cortex as well as a subsequent step (tethering or docking); (2) TBC1D4 functions during vesicle tethering and/or docking, and in the absence of insulin, a block in tethering repels incoming vesicles. Both models could explain the reduction in GLUT4 in the cell cortex when cells overexpress TBC1D4-4P. TBC1D4 dissociates from GLUT4 vesicles following insulin stimulation but this is not required for GLUT4 translocation (Stöckli et al., 2008). TBC1D4 possibly stays on GLUT4 vesicles until docking and fusion are completed. Consistent with this, phosphorylated TBC1D4 is highly enriched at the plasma membrane in response to insulin (Ng et al., 2010), which suggests additional functions for TBC1D4 at the plasma membrane (Fig. 4). A role for TBC1D4 in budding of GLUT4 vesicles from either endosomes or TGN also cannot be excluded.

Rab GTPases are major regulators of vesicle transport (Zerial and McBride, 2001), and active GTP-bound Rabs mediate either vesicle budding, movement along the cytoskeleton, membrane tethering, docking or fusion. The sequential nature of these steps in vesicle transport means that individual Rabs acting in a coupled mechanism could control each step. Consistent with this, the human genome encodes more than 60 Rabs and Rab cascades, i.e. pathways in which multiple Rabs and Rab-GAPs act in series (Rink et al., 2005; Rivera-Molina and Novick, 2009). Together with the fact that GLUT4 traffics through multiple compartments, it is not surprising that many Rabs (Rab 4, Rab5, Rab8, Rab10, Rab11, Rab13, Rab14 and Rab31) have been implicated in GLUT4 translocation (Huang et al., 2001; Ishikura et al., 2007; Kaddai et al., 2009; Kessler et al., 2000; Lodhi et al., 2007; Sano et al., 2007; Sano et al., 2008; Sun et al., 2010; Vollenweider et al., 1997). TBC1D4 displays strong GAP activity toward Rab8, Rab10 and Rab14 (Miinea et al., 2005). Many of these Rabs have been found on GLUT4 vesicles, although in view of the multitude of compartments through which GLUT4 traffics, it is difficult to pinpoint their individual functions (Jedrychowski et al., 2010; Larance et al., 2005). Based on the literature, we suggest that Rab4, Rab5, Rab11, Rab14 and Rab31 regulate endosomal and/or TGN trafficking of GLUT4 whereas Rab8, Rab10 and Rab13 might be involved in a Rab cascade that regulates GSV exocytosis.

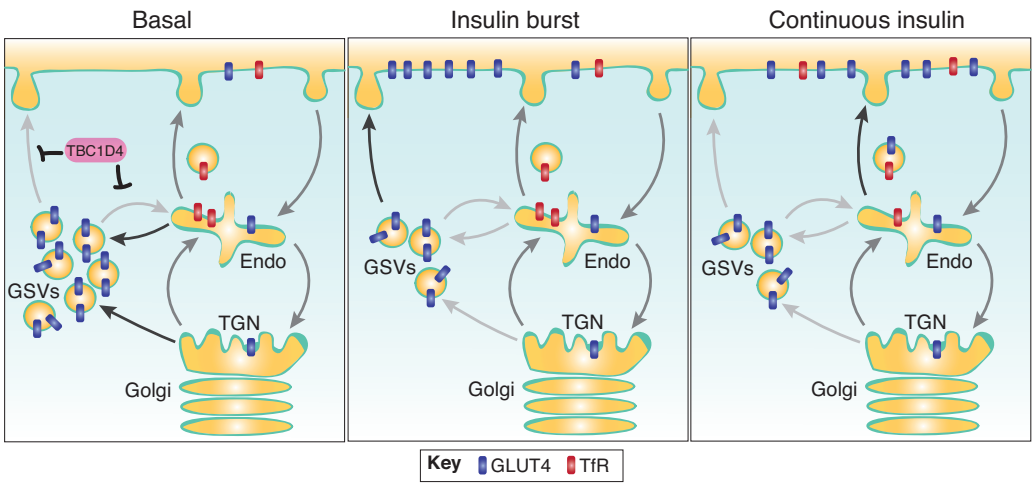



**Fig. 3. A model for GLUT4 trafficking.** In the absence of insulin (basal), a pool of GLUT4 is targeted to GSVs, which are derived from the TGN and/or endosomes. In the presence of insulin, these GSVs fuse directly with the plasma membrane in an initial burst (insulin burst). GLUT4 subsequently recycles through endosomes, the TGN and back to the plasma membrane through generic recycling compartments in the presence of insulin ('continuous insulin'). Following insulin withdrawal, GLUT4 traffics through the TGN to re-form GSVs. Arrows represent different trafficking rates: low (light grey), medium (dark grey), high (black). Endo, endosomes.

adipocytes (Thurmond et al., 1998). Munc18c is phosphorylated at Y521 in response to insulin (Aran et al., 2011; Jewell et al., 2011) and this has a role in insulin-dependent GLUT4 translocation. These tyrosine residues are located within a non-structured surface-exposed domain that has the potential to interact with other regulatory proteins and future studies examining the significance of this modification are necessary to precisely determine their role in insulin-stimulated GLUT4 translocation.

In neurons, the SNARE–SM complex works in collaboration with C2-domain-containing proteins, including synaptotagmin, DOC2b and Munc13 (Pang and Sudhof, 2010). This provides a link to the regulation of synaptic vesicle exocytosis through $Ca^{2+}$ because C2 domains bind to $Ca^{2+}$ and phospholipids. The recent identification of two C2-domain-containing proteins that might be involved in GLUT4 trafficking provides support for a role of $Ca^{2+}$ in this process. Indeed, previous studies have shown that insulin increases localised $Ca^{2+}$ concentrations beneath the plasma membrane in muscle cells (Bruton et al., 1999) and chelation of $Ca^{2+}$ with BAPTA inhibits insulin-dependent GLUT4 translocation in adipocytes (Whitehead et al., 2001). Extended synaptotagmin-like protein 1 (ESYT1), a synaptotagmin-like protein with five C2 domains, is targeted to the plasma membrane in adipocytes and phosphorylated by CDK5 in response to insulin (Lalioti et al., 2009). Double C2-like domains beta (DOC2B), a protein that contains two C2 domains, is localised to small punctate structures that are scattered throughout the cytoplasm in adipocytes; from these it translocates to the plasma membrane and binds to syntaxin-4 in response to insulin in a $Ca^{2+}$-dependent manner. Reducing DOC2B levels by RNA interference impairs insulin-dependent glucose transport in adipocytes (Fukuda et al., 2009). C2 domains insert into membranes and promote membrane curvature, thus further aiding fusion (McMahon et al., 2010). It has been proposed that C2-domain-containing proteins such as DOC2B are concentrated at membrane fusion sites by binding to SNARE complexes. The simultaneous binding of these proteins to membranes possibly on both sides of the cleft (i.e. GCV and the plasma membrane) might induce curvature, particularly in the plasma membrane, which is normally a flat membrane, and this, together with the energy provided by the assembling SNARE complex, is sufficient to induce fusion. It will be exciting to test elements of this model in the context of GLUT4 trafficking.

## Signals that regulate exocytosis

As discussed in the previous section, insulin leads to the translocation of GLUT4 to the plasma membrane. Here we discuss how this is achieved by focusing on the molecules that might coordinate the change in GLUT4 trafficking in response to insulin stimulation. We confine our discussion principally to the AKT pathway because, unlike other potential pathways, activation of AKT alone is sufficient to stimulate GLUT4 translocation to the plasma membrane in a manner that resembles insulin (Kohn et al., 1998; Ng et al., 2008). This does not discount a role for other regulatory pathways, such as those involving PKC or TC10 and c-Cbl, but there is currently a lack of consensus concerning their involvement in GLUT4 trafficking.

AKT phosphorylates TBC1D4 at multiple sites, which results in 14-3-3 binding to TBC1D4 and the presumed inhibition of TBC1D4 GAP activity (also see Box 3) (Miinea et al., 2005; Ramm et al., 2006; Sano et al., 2003). This enables a large number of GCVs to move to, dock at and fuse with the plasma membrane. Knockdown of TBC1D4 increases the level of GLUT4 at the cell surface by causing GTP loading of a cognate Rab, which regulates trafficking to and fusion with the plasma membrane (Eguez et al., 2005; Larance et al., 2005). By contrast, overexpression of a constitutively active TBC1D4 phosphorylation mutant blocks insulin-stimulated GLUT4 translocation (Sano et al., 2003).

Insulin-dependent phosphorylation of a range of other molecules also has a key regulatory role in GLUT4 translocation, namely ESYT1 and TC10 phosphorylation by CDK5 (Lalioti et al., 2009; Okada et al., 2008); MYO1C and MYO5 phosphorylation by CamKII (Karcher et al., 2001; Yip et al., 2008); RIP140 and SEC5 phosphorylation by PKC (Chen et al., 2011; Ho et al., 2009); Munc18c phosphorylation by the insulin receptor (Aran et al., 2011; Jewell et al., 2011), and PIKFYVE, AS250 and MYO5 phosphorylation by AKT (Berwick et al., 2004; Gridley et al., 2006; Yoshizaki et al., 2007). Because AKT activation alone is

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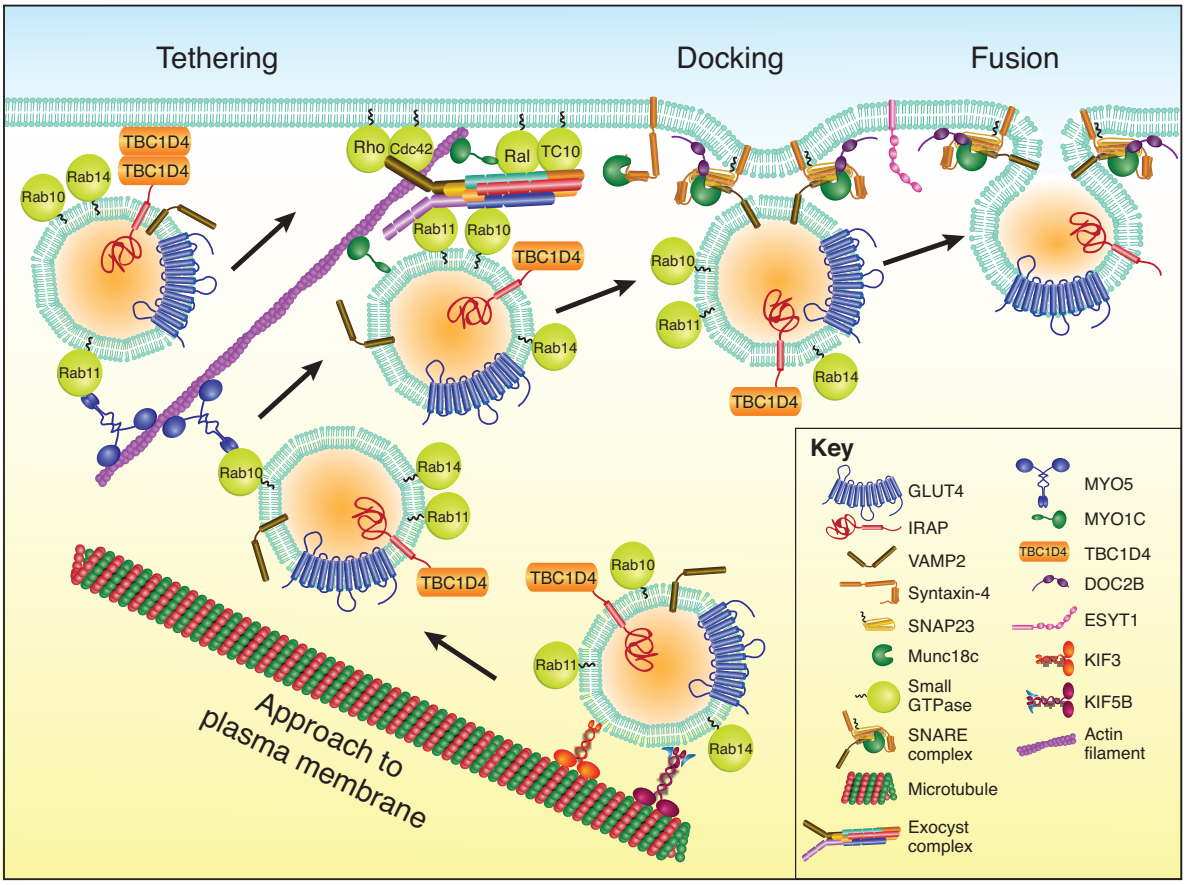


**Fig. 4. Overview of the steps involved in GLUT4 trafficking at the plasma membrane.** GSVs interact with a host of molecules as they are delivered to the cell periphery and tethered, docked and fused at the plasma membrane. GSVs approach the plasma membrane where they are tethered by actin, the exocyst or TBC1D4, or a combination of these in parallel or in series. The vesicle docks with the plasma membrane as the ternary complex is formed between VAMP2 on the GSV and syntaxin-4 and SNAP23 on the plasma membrane. This complex can then facilitate fusion of a GSV with the plasma membrane. The Rab proteins shown on GSVs in this figure were identified on GSVs in at least two separate proteomic studies (see www.jameslab.com.au/Contentpages/DataResources/GSVProteome.shtml).

sufficient to recapitulate insulin-induced GLUT4 translocation, it is possible that AKT-independent signalling pathways are passive consequences of the translocation process rather than active drivers (Ng et al., 2008). For example, if a GSV passes a crucial checkpoint such as the phosphorylation of TBC1D4 by AKT, the remaining regulatory steps might occur automatically, simply by virtue of being in the right place at the right time. In addition to activating GSV exocytosis, insulin also accelerates the exocytosis of endosomes, which explains the insulin-dependent increase in surface levels of endosomal proteins (Piper et al., 1991; Tanner and Lienhard, 1987; Volchuk et al., 1995; Wardzala et al., 1984). Regulation of endosomal recycling does not involve TBC1D4 (Brewer et al., 2011), but might require an alternative – but yet undefined – AKT substrate.

## Future perspectives

Considerable progress in our understanding of GLUT4 trafficking has been made over the past decade. GLUT4 is sorted into GSVs. These vesicles sequester GLUT4 away from the plasma membrane in the absence of insulin through a repulsion mechanism and they undergo rapid exocytosis in response to insulin in a burst-like manner. In the continuous presence of insulin, GLUT4 continues to recycle back to the plasma membrane, possibly through endosomes. AKT is the principal insulin-regulated signal transducer for GLUT4 translocation and several steps in the GLUT4-trafficking pathway are regulated by insulin, including the approach, tethering, docking and fusion of vesicles at the plasma membrane. Several AKT substrates, including MYOV, AS250, TBC1D4 have been implicated in GLUT4 trafficking.

These observations provide a clearer blueprint for future studies in this area. The first challenge is to define which of the many steps that govern GLUT4 trafficking is the major rate-limiting step for GLUT4 translocation and therefore glucose uptake. The second is to delineate the molecular components that choreograph this step and then pinpoint those components that are regulated by insulin. It will be essential to further develop high-resolution methods to distinguish between different GLUT4 compartments as well as the individual steps that are involved in the movement of these compartments to the plasma membrane. With this information in hand, it should be feasible to revisit disease states to determine whether this particular step and the molecules that underpin its regulation, contribute to defective GLUT4 translocation in muscle and fat cells and to insulin resistance. The recent realisation that insulin resistance is selective for specific actions of insulin gives rise to the possibility that GLUT4 translocation stands at the apex of the development of metabolic disease.

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