Building GLUT4 Vesicles: CHC22 Clathrin’s Human Touch

Gwyn W. Gould,1,* Frances M. Brodsky,2,* and Nia J. Bryant3,*

Insulin stimulates glucose transport by triggering regulated delivery of intracellular vesicles containing the GLUT4 glucose transporter to the plasma membrane. This process is defective in diseases such as type 2 diabetes (T2DM). While studies in rodent cells have been invaluable in understanding GLUT4 traffic, evolutionary plasticity must be considered when extrapolating these findings to humans. Recent work has identified species-specific distinctions in GLUT4 trafficking, notably the participation of a novel clathrin isoform, CHC22, in humans but not rodents. Here, we discuss GLUT4 sorting in different species and how studies of CHC22 have identified new routes for GLUT4 trafficking. We further consider how different sorting-protein complexes relate to these routes and discuss other implications of these pathways in cell biology and disease.

GLUT4 Trafficking in Health, Disease and Deep Time

GLUT4 is expressed predominantly (but not exclusively) in cells that exhibit insulin-stimulated glucose transport—adipocytes, and skeletal and cardiac muscle. When blood sugar levels rise, insulin released from the pancreas mobilises GLUT4 to the surface of these cells and elevates glucose transport into muscle and fat, lowering plasma glucose levels [1–3]. This insulin-responsive pathway is active in many vertebrate species where it is central to the maintenance of plasma glucose levels around a set-point, ensuring the essential constant supply of glucose to the brain and exercising muscles, balanced by the need to store excess as glycogen and fat. The required balance between these needs varies dramatically in vertebrate phylogeny, as does nutritional behaviour, which is also influenced by environmental niche. Thus, the insulin-responsive pathway shows evidence of selective pressure by nutrition and consequent variation in molecular details of how it regulates glucose balance at the cellular level [4]. For example, cave-dwelling fish have altered insulin receptors compared with their surface-dwelling counterparts to be able to handle a low carbohydrate diet [5]. In fact, nutritional variation as a strong evolutionary driver [6] can be compared with pathogen-driven selection of molecular variation in the immune response, for which there are many cited differences between humans and murine models, though effector processes are shared [7]. The discovery that the human GLUT4 pathway comprises molecular components that are not present in some other vertebrate species [4,8,9], including widely used rodent models, is therefore not surprising. Consequently, to understand human disease arising from malfunction of this pathway, we must consider both the commonalities and differences between GLUT4 membrane traffic in humans and model species, as well as genetic differences between human individuals.

Analysis of GLUT4 traffic in rodents has mapped routes shared in all species studied to date and laid the groundwork for understanding the basic pathway. In the absence of insulin, GLUT4 is sequestered in tubules and vesicles in the cytosol collectively referred to as the GLUT4 storage compartment (GSC; Box 1) [2,3]. GLUT4 is further sorted into ‘specialised’ insulin-responsive vesicles (IRVs), which are selectively mobilised to the cell surface upon insulin binding to its receptor [1–3].

**Highlights**

- Insulin stimulates glucose transport in fat and muscle cells by triggering the regulated delivery of GLUT4-containing IRVs to the cell surface.
- GLUT4 trafficking in human cells involves the noncanonical clathrin isoform CHC22 that sorts GLUT4 to the GSC.
- CHC22 clathrin is not expressed in rodents where different mechanisms underlie GLUT4 trafficking.
- New work has shown that CHC22-clathrin directs newly synthesised GLUT4 from the ER–Golgi intermediate compartment to the GSC without transit through the Golgi, in addition to mediating endosomal sorting of GLUT4.
- CHC22 clathrin interacts with p115, sortilin, IRAP, and GGA2 to mediate these distinct trafficking pathways.
- CHC22 clathrin enhances GLUT4 sequestration in human cells and is crucial for insulin-stimulated glucose transport.
- The newly identified CHC22-dependent pathway from the ERGIC could also operate in other cell types to control the delivery of cargo to specialised organelles.

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1Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, UK
2Division of Biosciences, University College London, London WC1E 6BT, UK
3Department of Biology and York Biomedical Research Institute, University of York, Heslington, York YO10 5DD, UK

*Correspondence: gwyn.gould@strath.ac.uk (G.W. Gould), f.brodsky@ucl.ac.uk (F.M. Brodsky), and nia.bryant@york.ac.uk (N.J. Bryant).
GLUT4 recycles between the cell surface and intracellular compartments, with the IRVs thought to be a specialised subendosomal compartment. However, key machinery involved in IRV formation is found in a number of cell types, including neurons [10,11], hepatocytes [12,13], and dendritic cells [14,15], suggesting that IRV-like vesicles are likely present in many cell types [16–18]. Adipocytes and muscle cells expand this compartment and so are uniquely endowed with an ability to rapidly modulate glucose transport in response to insulin (cardiac and skeletal muscle, fat) or exercise/contraction (cardiac and skeletal muscle) [19].

Newly synthesised GLUT4 may access IRVs from their site of synthesis while pre-existing GLUT4 may reach IRVs via endocytic retrieval from the cell surface involving the trans-Golgi network (TGN, see Glossary) and endosomal sorting. These likely have different regulatory mechanisms. GLUT4 has a half-life of 50 h in the absence of insulin in murine adipocytes [20], thus GLUT4 trafficking itineraries.

Box 1. Nomenclature

| Acronym | Definition |
|---------|------------|
| GSC     | All intracellular vesicles and tubules that contain GLUT4 – hence the GSC – this includes GLUT4 in endosomes, the TGN and the compartment targeted by CHC22 in humans. IRVs are regarded as a separate compartment. The GSC is likely comprised of further subcompartments not considered here for brevity [3,77,78]. |
| IRV     | Population of vesicles derived from the GSC that respond acutely to insulin by mobilisation to the cell surface. The general view is that these vesicles are depleted of endosome or Golgi markers. |
| GSV     | GLUT4 storage vesicles; this term is a source of confusion – some have used this to define a population of GLUT4 vesicles akin to IRVs. Others have used this with the same meaning as the GSC (above). For clarity, we use either GSC or IRV and avoid the use of GSV. |

Impaired insulin-stimulated delivery of GLUT4 to the plasma membrane (PM) of muscle and fat results in defective blood glucose homeostasis – a characteristic and defining feature of insulin resistance and T2DM. Evidence suggests this is a result of defective intracellular sorting of GLUT4 in patients with T2DM and obesity [3,21] (Box 2). Here, we summarise the recent data identifying a novel trafficking route followed by GLUT4 in human tissues to place this in the context of previous rodent studies, consider the composition of protein complexes that control GLUT4 traffic in humans and rodents, and re-examine existing data through the prism of new studies on GLUT4’s trafficking itinerary.

Human GLUT4 Sorting Needs a Second Coat

GLUT4 traffic is mainly studied either in primary rat or murine 3T3-L1 adipocytes with high GLUT4 expression and a large insulin response, or the rat L6 myoblast cell line transfected with human GLUT4 [22,23]. In all these cells, after insulin-mediated delivery to the cell surface (and in the absence of insulin), GLUT4 is internalised from the PM by clathrin-dependent endocytosis. Endosomal sorting and transport through the TGN then traffics GLUT4 into IRVs [1–3]. Trafficking through multiple endosomal compartments ultimately sequesters GLUT4 into specialised IRVs [24,25]. Both clathrin [25,26] and Golgi-localised γ-ear-containing, ARF-binding protein-2 (GGA2) clathrin adaptors [27,28] have been implicated in intracellular GLUT4 sorting in rodent cells.

Rodent cells have a single clathrin heavy chain isoform, CHC17, whereas humans express CHC17, forming the ubiquitous clathrin responsible for receptor-mediated endocytosis and other

Glossary

CHC17: the ubiquitous clathrin heavy chain isoform that plays a key role in endocytosis and other membrane trafficking pathways. CHC17 triskelion assemble into a polyhedral coat that captures cargo and facilitates the formation of endocytic vesicles at the cell surface or transport vesicles, for example, at the trans-Golgi network. CHC22: a noncanonical clathrin heavy chain isoform, expressed in some vertebrates, which has distinct biochemical and regulatory mechanisms. CHC22 forms triskelia but does not support endocytosis or vesicle formation from the PM. Clathrin triskelion: play a major role in the formation of coated vesicles during membrane traffic. Clathrin has a triskelion structure comprised of three clathrin heavy chains (CHC17 or CHC22). CHC17 clathrin also comprises light chain subunits that do not associate with CHC22.

Endoplasmic reticulum–Golgi intermediate compartment: a cellular sorting station that mediates trafficking between the ER and the Golgi. ER-derived cargo is trafficked from ER exit sites to the ERGIC in a COPII-dependent step and from the ERGIC is then delivered to the Golgi. The ERGIC is thus a key compartment in the early secretory pathway. The ERGIC also gives rise to membrane that forms autophagic vesicles diverted from the classical secretory pathway.

Golgi-localised γ-ear-containing, ARF-binding protein2: GGA family proteins are coat proteins that regulate the traffic of proteins in the endosomal compartment, and between the trans-Golgi network and the lysosome. They can bind ubiquitinated proteins. GGA2 is one family member.

Insulin-responsive aminopeptidase: a member of the zinc-dependent membrane aminopeptidases and a major constituent of the GSC and IRVs. Like GLUT4, IRAP exhibits robust insulin-stimulated translocation to the cell surface. The aminopeptidase activity does not appear to be essential for GLUT4 trafficking, but IRAP is a key mechanistic player in the biogenesis of the GSC/IRVs. IRAP is a type 2 integral membrane protein.

Sortilin: encoded by the SORT1 gene is a type 1 membrane glycoprotein and a member of the vacuolar protein sorting 10 protein family of sorting receptors. It
Box 2. GLUT4 in T2DM and Obesity

Insulin resistance in obesity and T2DM is characterised by decreased insulin-stimulated glucose transport and metabolism in adipocytes and skeletal muscle (and by impaired suppression of hepatic glucose output). Hence, many studies have sought to define potential sites of aberrant control of GLUT4 in human disease and in transgenic models of disease.

GLUT4 is selectively downregulated in adipocytes of T2DM patients, which leads to systemic insulin resistance [73]. This is mimicked in adipose-specific GLUT4 knockout mice [80]; similarly, adipose-specific overexpression of GLUT4 lowers fasting blood glucose levels and enhances glucose tolerance [81]. Studies in human muscle also point to defective GLUT4 sorting as a key feature of T2DM [8,9]. Defective GLUT4 sorting is also important in obesity. Adipose tissue-specific Ankyrin B knockout mice develop obesity [83]. Adipocytes from these animals exhibit increased lipid accumulation, increased glucose uptake, and impaired endocytosis of GLUT4. This is a consequence of Ankyrin B binding directly to GLUT4 and CHC17 clathrin, promoting their association. Ankyrin B variants that fail to restore normal lipid accumulation and GLUT4 localization in adipocytes are present in 1.3% of European Americans and 8.4% of African Americans and are candidates for contributing to obesity susceptibility in humans [83].

Maianu et al. examined the subcellular distribution of GLUT4 and IRAP in subcutaneous fat from control and T2DM patients [84]. T2D adipocytes exhibited an almost complete loss of insulin-stimulated glucose transport. Accompanying this impairment, the authors found that in the absence of insulin the subcellular distribution of IRAP was altered in T2DM – more IRAP was found at the PM and in dense membranes compared with controls. Also, the magnitude of insulin-stimulated IRAP translocation to the PM was impaired in T2DM. This was accompanied by a loss of insulin-stimulated GLUT4 translocation and a marked depletion of GLUT4 levels [84].

To fully understand GLUT4 trafficking in T2DM, future studies must consider the role of proteins such as CHC22 clathrin, sortilin, and p115 and the relative balance of trafficking routes (see Figure 1 in main text) to gain a full and clear understanding of potential sites of deficit in disease.

classical clathrin-dependent pathways, and an additional heavy chain isoform CHC22 that is 85% identical to CHC17 [9]. CHC17 and CHC22 form distinct clathrin triskelia [29], and CHC22 is highly expressed in human myocytes and also present in adipocytes. CHC22 associates with proteins previously shown to be involved in GLUT4 sorting (including GGA2 and the vSNARE vesicle-associated membrane protein (VAMP)2) [9]. In muscle sections from patients with insulin-resistant T2DM, CHC22 and GLUT4 compartments are considerably expanded compared with nondiabetic controls and the extent of overlap between CHC22 and GLUT4 is significantly greater than localisation of CHC17 to this expanded GSC [9]. CHC22 was found to mediate the retrograde traffic of GLUT4 from endosomes into the GSC but not PM endocytosis [29], suggesting that CHC22 participates in the regeneration of IRVs after their uptake following insulin-stimulated delivery to the PM [8,9]. These data on CHC22 in GLUT4 traffic emerging from our laboratory are complemented by the observation from Nahorski and colleagues of a potential role for CHC22 during early brain development that suggested a similar function in endosomal sorting in human neuronal cells [10,11]. However, studies from our laboratory [30] have now revealed a more complex role for CHC22 in GLUT4 traffic.

CHC22 depletion from human myocytes results in their inability to sequester GLUT4 and respond to insulin despite the presence of CHC17 at roughly ninefold higher levels [8,9]. This is surprising, as both CHC22 and CHC17 mediate retrograde traffic from endosomes and suggests that CHC22 plays a distinct, additional role in GLUT4 trafficking in human cells that cannot be recapitulated by CHC17, and by inference suggests that CHC22 mediates a GLUT4 trafficking step distinct from retrograde trafficking from endosomes to the GSC. This is likely a pathway that specifically enhances GLUT4 traffic to the GSC, as transgenic overexpression of CHC22 in mouse muscle caused accumulation of GLUT4 in enlarged intracellular structures that also contained the insulin-responsive aminopeptidase (IRAP), a known GSC component (Box 3) [9]. Reinforcing the parallels with T2DM patients, these mice exhibited hyperglycaemia with ageing [9]. Such results prompted an attempt to re-address CHC22-mediated GLUT4 trafficking pathways in human cells with potential to shed new light on the aetiology of T2DM.
Our further studies of the role of CHC22 in membrane trafficking uncovered a second route for GLUT4 trafficking in human cells involving sorting at the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) and has begun to define key molecular complexes that regulate GLUT4 sorting at different locations within the cell [30]. This model of GLUT4 trafficking is presented in Figure 1 (Key Figure).

Experiments utilising the RUSH (retention using selective hooks [31]) approach to synchronously release GLUT4 (and GLUT1 as a control) from the ER showed that after initially colocalising in the perinuclear region, some GLUT1 vesicles traffic towards the PM while GLUT4 remains intracellular [30], consistent with earlier studies in rodents which argued that newly synthesised GLUT4 traffics directly to the GSC without accessing the PM [32]. Localising GLUT4 and CHC22 relative to organelle-specific markers using both conventional and super-resolution immunofluorescence microscopy revealed extensive overlap of CHC22 with ERGIC53 and p115, and suggested that GLUT4 remains longer in early secretory pathway compartments after biosynthesis than the GLUT1 that traffics constitutively to the PM [30]. This CHC22-positive early secretory compartment was observed adjacent to structures labelled for syntaxin (Sx)6 (Box 3), suggesting...
Key Figure
Role of CHC22 in GLUT4 Trafficking in Human Cells

**Figure 1.** Shown is a model based upon a scheme agreed by 15 groups in the GLUT4 field at a recent meeting (https://www.ucl.ac.uk/research/domains/food-metabolism-and-society/events/glut4-traffic-map-workshop). Newly synthesised GLUT4 traffics from the ER into the ERGIC. A recently identified trafficking route (Route 1) delivers GLUT4 directly into the GSC; this involves a complex between IRAP and p115 that recruits CHC22 clathrin, which sequesters GLUT4 (Figure 3, Complex 1). This allows sorting of GLUT4 into the GSC and ultimately IRVs. Insulin promotes delivery of IRVs to the cell surface (green arrows). GLUT4 reinternalisation involves endocytosis by CHC17 clathrin from the cell surface and the delivery of GLUT4 into the endosomal system (orange arrows). GLUT4 can re-enter the GSC via Route 2. Here, a complex between sortilin and the CHC22 clathrin adaptor GGA2 promotes the assembly of a CHC22 coat (Figure 3, Complex 2). Route 2 may involve the adaptor protein AP1 that also interacts with CHC22. This retrograde trafficking route allows replenishment of the GSC/IRV pools. The proposed intracellular site for ubiquitin-dependent sorting of GLUT4 (Figure 2) is indicated by the orange star. Abbreviations: ERGIC, endoplasmic reticulum–Golgi intermediate compartment; GGA2, Golgi-localised, γ-adaptin ear-containing, ARF-binding 2; GSC, GLUT4 storage compartment; IRAP, insulin-responsive aminopeptidase; IRV, insulin-responsive vesicle.
that newly synthesised GLUT4 is sequestered close to but distinct from structures accessed by retrograde trafficking of GLUT4 from endosomes [8,30]. Hence, CHC22 appears to mediate two aspects of GLUT4 sorting in human cells (Figure 1) – retrograde sorting from endosomes, and diversionary traffic of newly synthesised GLUT4 from the ERGIC to the GSC, while CHC17 mediates internalisation of GLUT4 from the PM in human cells.

Further demonstration that CHC22 mediates membrane trafficking emerging from the ERGIC came from studies using *Legionella pneumophila*; a bacterium that co-opts membrane from the ERGIC to create an *L. pneumophila*-containing vacuole (LCV) for replication and avoid the degradative environment of the endocytic pathway [33]. Upon infection with *L. pneumophila*, the LCV is enriched in CHC22, which is required for LCV formation. This indicates that CHC22 plays an active role in trafficking ERGIC membranes [30]. The LCV also acquires essential components of the GLUT4 pathway – IRAP, sortilin, and GGA2 (Box 3), consistent with CHC22 mediating the trafficking of proteins involved in formation of the GSC from the ERGIC [30].

Super-resolution microscopy also shows that the CHC22 compartment does not overlap with either the cis- or trans-Golgi markers GM130 or TGN46 in either HeLa or human muscle cells [30]. Importantly, depletion of the cis-Golgi tether GM130 has no effect on GSC formation or distribution or on insulin-stimulated trafficking of GLUT4, indicating that a Golgi bypass occurs in GLUT4 trafficking, defining a ‘direct-targeting’ pathway (Figure 1, Route 1). This previously unappreciated route on the GLUT4 SatNav must be integrated with what we already know about GLUT4 trafficking. Given that CHC22 is involved in sorting into the human GSC, its proposed molecular partners and how they relate to sorting mechanisms identified in rodent models is the focus of the next section.

**How to Make an IRV**

Investigators have sought to identify specific machinery that mediates GLUT4 intracellular sequestration into IRVs. One widely used approach has been proteomic analyses of purified GLUT4 vesicles from rodent cells. These studies have provided an extensive ‘parts list’ (Box 3). However, with the notable exception of TUG (Box 3) [34,35], none of the machinery identified appears to be either selective for GLUT4 or specific to GLUT4-containing cells. Furthermore, GLUT4 is intracellularly sequestered and exhibits insulin-dependent trafficking to the surface in all cell types studied, albeit with some variations [19]. Nevertheless, the molecules identified have contributed greatly to our knowledge of GLUT4 trafficking. Before integrating these molecules with the CHC22 studies, we shall consider how these proteins fit in GLUT4 trafficking.

**IRAP or Sortilin – Which Is the Master Regulator?**

IRAP is a major constituent of the GSC and IRVs and exhibits robust insulin-stimulated translocation to the PM [36]. Its aminopeptidase activity does not appear to be essential for GLUT4 trafficking, but IRAP is a key player in the biogenesis of the GSC/IRVs. Unlike GLUT4, IRAP is widely expressed, and appears in murine 3T3-L1 cells before differentiation into adipocytes. IRAP intracellular sequestration is enhanced during adipogenesis and occurs before GLUT4 is expressed [37]. In 3T3-L1 adipocytes, although IRAP knockdown had profound effects on GLUT4 traffic, GLUT4 knockdown does not affect IRAP trafficking, demonstrating that IRAP traffics independently of GLUT4. This suggests that IRAP is both cargo and a key regulator of the GSC/IRVs [38]. A different view was reached when GLUT4 was knocked out or overexpressed in transgenic mice. Jiang *et al.* observed impaired trafficking and insulin-stimulated translocation of IRAP in fat and muscle of GLUT4 knockout mice [39], and Carvalho *et al.* observed changes in IRAP levels that paralleled GLUT4 overexpression or knock-out [40]. This issue is returned to below.
A counter-view arises from studies of sortilin, an abundant component of the GSC (Box 3). Sortilin has a GGA-interaction motif and its role in IRV formation was studied because of its ability to recruit coat proteins. Huang et al. showed that translocation of GLUT4 expressed in mouse fibroblasts is enhanced by sortilin overexpression [41]. They argue this is because GLUT4 is more effectively sequestered into IRVs in the presence of sortilin. Similarly, forced expression of GLUT4 prior to induction of sortilin leads to rapid degradation of the transporter, whereas overexpression of sortilin increases formation of IRVs and stimulates insulin-regulated glucose uptake [28,42]. The Vps10 domain of sortilin directly binds the lumenal loop of GLUT4 and the C-terminal tail of sortilin binds retromer (see below), suggesting that sortilin rescues GLUT4 from lysosomal degradation and may function by linking GLUT4 directly to the retromer sorting machinery [43]. Consistent with this, the endosome/Golgi retrieval system develops in response to sortilin expression and requires the interplay of Sx6, retromer and golgin-97 [44]. Such studies place sortilin at the heart of GLUT4 trafficking, but it is important to note that in these studies, IRAP is still present.

Debate about the importance of the sortilin- and IRAP-dependent GLUT4 trafficking pathways has been provoked by a recent study in murine cells showing that IRAP trafficking is sortilin independent because IRAP can bind retromer directly whereas GLUT4 can only bind via sortilin [45]. This study found that CRISPR knockout of IRAP in 3T3-L1 adipocytes did not affect either GLUT4 levels or translocation – in contrast to data using knockdown or mutational approaches and more in-line with data from transgenic GLUT4 knockout mice [45]. Such distinctions may arise as a consequence of the methodological approaches used. Regardless, these data indicate that both IRAP and sortilin are crucial for biogenesis of IRVs, but still begs the question of where and how IRAP and sortilin function in GLUT4 trafficking.

IRAP binds to p115, a protein found in the early secretory pathway (Box 3) [46]. Expression of a fragment of p115 encompassing the IRAP interaction domain had a dominant negative effect on GLUT4 translocation in murine cells [46]. Such data, together with studies revealing an interaction between IRAP and insulin signalling proteins such as the RabGAP AS160 support the notion that IRAP may act to scaffold key signals for GLUT4 trafficking [47]. A further clue was provided by the observation that IRAP binds tankyrase (TNKS; Box 3), a poly-ADP-ribosyltransferase [48,49]. Knockdown of TNKS or inhibition of TNKS activity in 3T3-L1 adipocytes impaired GLUT4 translocation by mis-sorting GLUT4 away from IRVs [49]. Treatment of rat L6 muscle cells with the TNKS inhibitor XAV939 resulted in insulin resistance and a decline in proteins that regulate GSC formation, including sortilin [50]. Hence in rodents, TNKS appears to be a key player in the biogenesis of IRVs.

Ubiquitin-Driven Decisions

Ubiquitination of GLUT4 (Ub-GLUT4) is essential for delivery of GLUT4 into IRVs, the same sorting step that requires GGA proteins, raising the possibility that clathrin/GGA interactions may play a role in sorting Ub-GLUT4: this could be either CHC17 or CHC22, depending on species [27,51]. Ubiquitination normally signals proteins for degradation – so how does GLUT4 escape this fate? Our recent work has suggested this may be a consequence of an association between TNKS and the deubiquitination enzyme USP25, which has also been implicated in regulating other facets of GSC stability [52]. In this model Ub-GLUT4 is sorted into IRVs whereupon the presence of USP25 (bound via TNKS to IRAP) de-ubiquitinates GLUT4 and allows GLUT4 accumulation in IRVs rather than its degradation. Hence, cycles of ubiquitination/deubiquitination serve to correctly transition GLUT4 from the endosomal system towards IRVs, with IRAP playing a central role as a scaffold/organiser via TNKS and USP25 (Figure 2) [52].

A splice-variant of USP25, Usp25m, has also been shown to regulate GLUT4 translocation via the cleavage of TUG protein (tether containing a UBX domain, for GLUT4) (Box 3), which traps
GLUT4 intracellularly in unstimulated 3T3-L1 adipocytes and releases GLUT4 upon insulin stimulation. The depletion of Usp25m reduces GLUT4 abundance and impairs translocation to the cell surface, and is thought to disrupt trafficking to IRVs and promote GLUT4 degradation in lysosomes [53].

TNKS is further implicated in GLUT4 trafficking by Guo et al.’s identification of a ternary complex comprising Axin/TNKS and the kinesin motor protein KIF3A, which they suggest is required for insulin-stimulated GLUT4 translocation. They showed that insulin inhibits ADP-ribosylation activity of TNKS and decreases ADP ribosylation and ubiquitination of both Axin and TNKS [54], supporting an additional role for ubiquitin signalling in GLUT4 trafficking.
Retromer – Fine-Tuning GLUT4 Trafficking Decisions?

The observation that the adaptor AP1 and retromer play opposing roles in trafficking of sortilin between the Golgi apparatus and lysosomes was one of the first hints that retromer could function in trafficking pathways germane to GLUT4 [55]. The retromer subunit Vps26 is associated with T2DM in South Asian populations [56], and genome-wide association and other studies have further piqued interest in retromer in the context of diabetes [57,58]. Using a human adipocyte cell line, Yang et al. showed that retromer components Vps26, Vps35, and SNX27 colocalise with intracellular GLUT4 and exhibit insulin-dependent translocation to the PM [59]. Knockdown of retromer does not affect insulin signalling but decreases the stability of sortilin and GLUT4, blocks their entry into IRVs, and reduces insulin-stimulated glucose transport [59]. Such observations indicate that the retromer has an important role in both formation and stability of the GSC.

In support of this, Ma et al. examined the mechanism by which long-term insulin treatment impairs GLUT4 sorting and downregulates GLUT4 levels in 3T3-L1 adipocytes. They found that insulin acutely dissociates retromer from internal membranes by disrupting Vps35/sortilin interactions, an effect mediated by the protein kinase CK2 [60]. Expression of a Vps35 mutant in which the CK2 phosphorylation motif is mutated (Vps35-S7A) attenuates GLUT4 downregulation in response to long-term insulin treatment. Importantly, insulin-generated oxidative stress changes the direction of GLUT4 traffic to lysosomes by inhibition of retromer function via CK2 [60]. These data indicate that retromer is a crucial component in regulating GLUT4 and GSC stability and may help fine-tune GLUT4 sorting under certain cellular conditions.

To integrate the recent findings regarding the role of CHC22 in GLUT4 traffic with these studies on players in GSC formation, we now address potential links between CHC22, sortilin, and IRAP.

Complex Complexes of CHC22, Sortilin, and IRAP

The localisation of CHC22 to the early secretory pathway, together with its role in retrograde trafficking from endosomes to the GSC, prompted us to examine the complexes formed by CHC22, p115, IRAP, and sortilin in human cells and to test the hypothesis that different CHC22 subcomplexes may exist to mediate trafficking from the ERGIC to the GSC and from endosomes to the GSC [30]. CHC22, but not CHC17, coimmunoprecipitates p115, consistent with the proposed role of the ERGIC in GLUT4 traffic. CHC22 also preferentially coimmunoprecipitates GLUT4, GGA2, and sortilin; thus, CHC22 forms complexes with molecules from both the early secretory pathway and the GLUT4 retrograde transport pathway, consistent with the dual role of this coat (Figure 1). siRNA has been used to assess the impact of CHC22 knockdown on molecules involved in GSC formation. Depletion of CHC22 reduces GLUT4, sortilin, and GGA2 levels with no obvious change in either IRAP or p115 levels. Depletion of p115 reduces cellular levels of IRAP but not GLUT4, sortilin, or CHC22 (knockdown of CHC17 is without effect on any of the GLUT4 trafficking components) [30]. Based on these data, we propose the idea of two distinct CHC22-containing complexes, one of CHC22/GGA2/sortilin/GLUT4 involved in retrograde sorting of GLUT4 to IRVs within endosomes, and another of CHC22/p115/IRAP/GLUT4 that mediates the sorting of newly synthesised GLUT4 from the ERGIC (Figure 3). Further work is needed to define the relative prevalence and composition of these complexes and identify their potential rodent counterparts.

We propose that in human cells CHC22/p115/IRAP interaction triggers the coalescence of a protein domain that captures GLUT4 for sorting to the GSC. Depletion of CHC22 or p115 in myocytes or HeLa cells results in dispersal of the GLUT4 perinuclear compartment and impaired insulin-stimulated GLUT4 translocation. By contrast, GLUT4 dispersal is not observed upon depletion of IRAP or sortilin, and GLUT4 translocation is unaffected [30]. This is distinct to data
obtained in rodent cells [41,42,49] and points to clear, species-associated differences in GLUT4 sorting pathways (Box 4). These data suggest that CHC22 and p115 are critical in the formation of the human GSC and argue that membrane traffic from the early secretory pathway direct to the GSC, bypassing the Golgi, is an important facet of human GLUT4 trafficking [30]. That expression of p115 fragments in murine cells interferes with GLUT4 translocation [46] and that Sec16a, another ERGIC-associated trafficking protein is associated with insulin-regulated GLUT4 trafficking [61] may indicate that the ERGIC-exit pathway operates at a low level, without a specific coat protein, in formation of the murine GSC. This suggests that the presence of CHC22 enhances the participation of this ‘direct-targeting’ pathway in the generation of the human GSC. Thus, pathways for GLUT4 targeting to the GSC/IRVs are shared between humans and rodents but due to the presence of CHC22, the ERGIC exit pathway has a more pronounced role in humans, and by inference, the pathway of endosomal sorting following PM recapture dominates in rodents (Box 4). This hypothesis of differential balance is consistent with the observation that when CHC22 is expressed transgenically in murine muscle, GLUT4 is oversequestered and GLUT4 levels on the sarcolemma are reduced compared with those in wild-type mice. The resulting hyperglycaemia fits with excessive intracellular targeting of GLUT4, changing the dynamic of the murine insulin response.

Figure 3. CHC22-Containing Complexes in GLUT4 Trafficking Routes. Shown are complexes of proteins that act with CHC22 to facilitate GLUT4 trafficking. Two distinct complexes have been proposed. Complex 1, assembles on the ERGIC and involves IRAP and p115. This mediates traffic of GLUT4 from the ERGIC to the GSC (Route 1 in Figure 1). Complex 2 drives retrograde sorting of GLUT4 from endosomes into the GSC (Route 2 of Figure 1), and comprises GLUT4, Sortilin, GGA2, and AP1 adaptor proteins. Note that CHC22 is assembled on the cytoplasmic face of the vesicles containing GLUT4. The proposed complexes are based on evidence from recombinant protein interactions, co-immunoprecipitation and coordinated regulation [30]. Abbreviations: ERGIC, endoplasmic reticulum–Golgi intermediate compartment; GGA2, Golgi-localised, γ adaptin ear-containing, ARF-binding 2; GSC, GLUT4 storage compartment; IRAP, insulin-responsive aminopeptidase.
Box 4. Of Transgenic Mice and Men – A Question of Balance

Understanding GLUT4 sorting/trafficking in disease is of considerable therapeutic importance; mis-sorting GLUT4 away from IRVs or mis-trafficking towards degradative compartments may underpin blunted insulin-stimulated glucose transport (Box 2). However, distinctions between GLUT4 traffic in human and rodents indicate that the relevance of transgenic mouse studies to human disease must be considered in the context of these and other species differences.

The reported distinctions in GLUT4 trafficking in human cells compared with rodents include CHC22 clathrin and Sx10, a SNARE involved in retrograde endosomal sorting which is absent from mice [8]. Depletion of Sx10 or CHC22 from human myoblasts results in GLUT4 dispersal, suggesting a connection between these proteins [8].

The demonstration that both human and rodent cells use retrograde trafficking to direct GLUT4 into the GSC indicates that this pathway is important [8,30]. Studies in murine 3T3-L1 adipocytes have shown that newly synthesised GLUT4 reaches the GSC before its insulin-dependent release to the PM, and that p115 is involved in this step [32,46,99]. Thus, in both humans and rodents, endocytic recapture and what we term ‘direct targeting’ are involved in GSC formation.

Humans use CHC22 as a coat for both pathways, but rodents lack CHC22. We hypothesise that rodents rely on the coalescence of GLUT4, IRAP, and p115 in the ERGIC to segregate them from other protein cargo that traffics directly to the PM. This process may be less efficient without a coat, placing a greater emphasis on efficient endosomal sorting to target GLUT4 to the GSC, and thus a greater reliance on sortilin and IRAP in murine cells [90]. The use of extremes (no or maximal insulin) may also overemphasise the role of endocytic retrieval in murine GLUT4 trafficking given that the need to effectively remove GLUT4 from the PM in the complete absence of insulin predicates an active and effective endosomal sorting mechanism for GLUT4 traffic to IRVs. Thus, differences between experimental approaches used to study GLUT4 translocation in each species, as well as the fact that humans have an added stable and efficient coat for both targeting pathways to the GSC may explain why sortilin and IRAP knockdown has (relatively) less effect on GSC formation in human cells compared with murine cells.

It should also be noted that the half-life of GLUT4 in human tissues has not been established. Studies suggest a 50-h half-life in the absence of insulin in rodents [20], but shorter times have been observed in other circumstances, such as only 8 h in exercising dogs [100]. The relative contributions of Route 1 and Route 2 (see Figure 1 in main text) to replenishing IRVs thus needs to be carefully evaluated together with an analysis of GLUT4 half-life in human cells.

We argue that the CHC22-mediated sorting of GLUT4 should be considered as an example of species-associated specialisation, and not a point of divergence between two schools of thought. Studies in both mouse and human have revealed important arms of the GLUT4 pathway that operate in both species, but perhaps to different degrees.

CHC22 and GSC Machinery in Other Cell Types

Action potential firing recruits GLUT4 to the surface of hippocampal nerve terminals from a GSC-like compartment [62], and insulin recruits GLUT4 to the surface of specific brain regions [63–65]. Hence, correct sorting and packaging of GLUT4 into the GSC/IRVs is relevant beyond peripheral tissues [63–66]. IRAP is expressed in specific brain regions, including the hippocampus, neocortex, and motor neurons [63,67,69], sortilin is involved in chaperoning neuronal receptors [69] and a GSC-like compartment regulates somatostatin receptor storage in pituitary cells [70]. A GSC/IRV-like compartment may thus be present in many cell types to enable signal-mediated regulation of PM levels of key proteins [19]. CHC22 is expressed in the developing human brain, and Nahorski et al. identified a homozygous missense mutation in CHC22 in children unable to feel pain or touch [10]. They also showed that CHC22 affects neurotrophic signalling by regulating a novel lysosomal trafficking pathway that controls packaging of neuropeptides into dense core granules [10,11]. Hence, CHC22-dependent trafficking pathways may underpin a range of specific cellular functions. Although CHC22 protein levels are generally low except in muscle and adipose cells [8], CHC22 could still play a vital role in specialised tissue-specific trafficking.

IRAP and other GSC machinery play roles beyond GLUT4 trafficking. Dendritic cells use major histocompatibility complex class I molecules to present peptides derived from internalised antigens to cytotoxic T lymphocytes (crosspresentation) [15,71]. Strikingly, this process utilises peptide trimming by IRAP in a Rab14 compartment (Box 3) with characteristics similar to the GSC, suggesting that GSC-like compartments may be adapted for other cellular functions.
Supporting this, IRAP is highly expressed in mast cells where it localizes to intracellular vesicles containing VAMP3 and VAMP2, which have different markers than secretory granules [72]. Stimulation of mast cells by antigen/IgE complexes triggers rapid IRAP translocation to the PM [72]. Hence, IRAP may identify a combination of pathways and intracellular structures whose conserved mechanisms and regulatory framework can be adapted across multiple tissues. The role of CHC22 has not been investigated in these events, but the CHC22-dependent pathway emanating from the ERGIC may be involved in trafficking other cargo to similar GSC-like compartments in other cells.

**Do Hunter-Gatherers and Farmers Offer a Clue to a Modern Disease?**

This new insight into GLUT4 trafficking raises interesting questions regarding the role of this pathway in human glucose homeostasis. A step in this direction comes from our recent population genetic and phylogenetic analyses of the CHC22-encoding CLTCL1 gene [4]. Humans have maintained two high-frequency CLTCL1 allelic variants, encoding either methionine or valine at position 1316. These variants have subtle differences in their intracellular dynamics that translate to functional distinctions. Knockdown of CHC22 in HeLa cells (M1316 homozygous) impaired insulin-stimulated translocation of GLUT4; expression of CHC22-M1316, but not CHC22-V1316 restored insulin-stimulated GLUT4 translocation, arguing that the M1316 allelic variant is more effective at targeting GLUT4 to the GSC [4]. Notably, CHC22-V1316 can function to sequester GLUT4 in transgenic mice, so it is functional, just not effective in the HeLa cells, which achieve only a low level of GLUT4 translocation. To further probe the distinctions between these allotypes, cells were depleted of endogenous CHC22 and transfected with mCherry-GLUT4 plus either CHC22 allotype then divided into three populations expressing equivalently low, medium and high levels of the transfected CHC-GFP. Assessing the total GLUT4 content of the cells by mCherry fluorescence showed higher levels of GLUT4 in cells expressing CHC22-M1316-GFP, compared with cells expressing CHC22-V1316-GFP, at medium and high levels of CHC expression. This suggests that the CHC22-M1316 variant is more efficient than CHC22-V1316 in targeting GLUT4 to the GSC, thus preventing its degradation [4]. Conversely, the CHC22-V1316 coat is more dynamic, perhaps due to structural effects of the mutation on assembly, which would sequester GLUT4 less effectively. This may in turn make GLUT4 more available at the PM, potentially enhancing the threshold for glucose clearance.

CHC22-V1316 is more frequent in farming populations than in hunter-gatherers, suggesting a correlation with regular consumption of digestible carbohydrate [4]. Hence, ancestral human dietary change could have influenced selection of allotypes that affect the role of CHC22 in metabolism and have potential to differentially influence glucose clearance in response to insulin. This evolutionary perspective paves the way to examine how the CHC22 pathway may be modulated in insulin resistance/T2DM.

**Concluding Remarks**

The identification of an enhanced GLUT4 trafficking route in human cells and the synthesis of data implicating IRAP and sortilin in distinct GLUT4 trafficking steps provides a coherent model into which other factors such as TUG [34,73] and low-density lipoprotein receptor-related protein (LRP)1 [74], can be positioned. Many new questions arise (see Outstanding Questions): what are the relative contributions of these different routes into the GSC/IRVs? Does the balance between them change under different physiological conditions and in different species? Is it plausible that different physiological needs dictate this balance – might this be altered by exercise or prolonged high blood glucose? Glycosylation of GLUT4 is necessary for function [75] – begging the question of how GLUT4 moving from the ERGIC directly into IRVs acquires complex glycosylation modifications? What is the role of CHC22 itself – does it sequester GLUT4 cargo through specific adaptors?

**Outstanding Questions**

What are the relative contributions of Route 1 and Route 2 (Figure 1) to GSC/IRV formation in different species? How, if at all, does this affect GLUT4 half-life?

Given that glycosylation is important for GLUT4 trafficking, when and how does ‘Golgi-bypassed’ GLUT4 get glycosylated? Is glycosylation maturation more extensive in rodents than humans?

Are levels or function of CHC22 clathrin and the Golgi bypass trafficking pathway modulated in diseases such as T2DM or obesity?

What is the connection between the ERGIC-derived compartment and the pool of GLUT4 generated from the endocytic pathway?

How does insulin signalling intersect with the different sets of complexes shown in Figure 3?

How is the reversible addition of ubiquitin to GLUT4 achieved? Are Ub-GLUT4 levels altered in diabetes?

What is the molecular detail of the multiple interactions revealed in Figure 3 – which proteins bind whom and where? How does GLUT4 get packaged?

CHC22 clathrin and IRAP are present at variable levels in many cell types – what function do they fulfill in these cells and what other cargo traffic “like GLUT4” avoiding the conventional secretory or endocytic trafficking routes? And why?
What localises CHC22 to its site of function? And, what is the connection between the ERGIC-derived compartment and the pool of GLUT4 generated from the endocytic pathway? The answer to such questions will offer essential new insight into glucose homeostasis.

Future studies of how CHC22-dependent GLUT4 trafficking steps are modulated in diseases like diabetes will be informative. Analysis of the interplay between these different GLUT4 trafficking paths and insulin signalling has barely begun. The observation that IRAP translocation in other cell types is triggered by signalling pathways distinct from those used by insulin, and that IRAP interacts with key signalling proteins, including the RabGAPs Tbc1D1 and Tbc1D4 in peripheral tissues [47,76] opens yet further possibilities to fine tune GLUT4 traffic. We look forward to an exciting new period in GLUT4 biology.

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