

Structure and function of GLUT4's *N*-glycan: a novel therapeutic target for type 2 diabetes?

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Abstract

Type 2 diabetes (T2D) is a major healthcare challenge, characterised as dysregulated glucose homeostasis. The glucose transporter GLUT4 is key for maintaining healthy blood glucose levels. Insulin stimulates GLUT4's transport to the cell surface for glucose uptake into fat and muscle cells, a process which is defective in T2D. While GLUT4 has been intensely studied, little has been researched about the role of its single *N*-glycan. *N*-glycosylation is the post-translational addition of a carbohydrate chain to a specific consensus site on a protein, a modification which has a wide variety of functions. A potential function of GLUT4's *N*-glycan was identified in the regulation of GLUT4 trafficking. This project aimed to further characterise the function of GLUT4's *N*-glycan and elucidate its structure. This was done through a range of methods, including altering the *N*-glycan's structure with glycosylation inhibitors and blocking the binding of potential interactors. A HA-GLUT4-GFP HeLa cell line was used, which demonstrates insulin-responsive trafficking of GLUT4 comparable to adipocytes. Using the HA and GFP tags to measure surface GLUT4 as a proportion of whole GLUT4 revealed two roles for GLUT4's *N*-glycan in regulating GLUT4 trafficking – one in the intracellular sequestration of GLUT4, and the other in cell surface retention. In addition, analysis of steady state GLUT4 suggested a third role in the regulation of GLUT4 degradation. The structure of GLUT4's *N*-glycan was shown to be crucial to each of these functions. Monosaccharide composition analysis revealed the presence of branched poly lactosamine in addition to an unidentified monosaccharide not previously discovered in mammalian cells.

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Author's declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as references.

1 General Introduction

1.1 Diabetes

One of the major healthcare crises facing the global population is the rapidly rising number of people living with diabetes. In 2021, around 537 million people were living with diabetes, with that number expected to rise by around 45% over the next 20 years (Sun et al., 2022). Not only is diabetes itself a challenging disease to manage, involving constant maintenance of whole body glucose levels, it can cause a multitude of complications including cardiovascular diseases, kidney damage, eye diseases, damage to the nervous system and/or damage to the vascular system. Furthermore, in countries which do not have free healthcare, the constant requirement for insulin and/or other medications can be a financial burden, causing particular pressure in poorer communities.

Diabetes is defined as a disease where the homeostasis of whole body glucose is disrupted by the inability of the body to produce enough insulin, or to respond appropriately to insulin. Insulin is essential for regulation of glucose levels, as it plays a variety of roles in muscle cells, fat cells and the liver. These roles include promoting glucose transport from the blood into muscle cells and adipocytes, activating glycogen synthesis and decreasing gluconeogenic gene expression in the liver (Petersen and Shulman, 2018). When glucose levels are not properly controlled, it can cause widespread disruption within the body.

While there are many variations of the disease, the majority of cases fall into either type 1 or type 2 diabetes (T1D and T2D respectively). T1D is typically a more aggressive disease resulting from the loss of the insulin-secreting function of pancreatic beta cells, and while it

can develop at any age, it is more commonly diagnosed in childhood or adolescence. While there is no cure, it can be managed through daily insulin injections and glucose monitoring.

By far the more prevalent form of diabetes is T2D, accounting for around 90% of total diabetes cases. T2D occurs when fat and muscle cells become unresponsive to insulin, requiring a larger amount of insulin to be secreted until the demand exceeds the ability of the pancreatic beta cells to supply. This results in the inability of cells to absorb glucose from the blood, causing hyperglycaemia. T2D has a slower onset than T1D, and although the causes are not fully known, there is a strong link with obesity, as environmental influences such as poor diet and low exercise levels are known to be risk factors (as reviewed in Kahn et al, 2008). Other risk factors include age, ethnicity and genetic history. While similarly to T1D there is no known cure, T2D can be prevented and/or controlled through healthy diet and regular exercise, and in some cases has been brought into remission by such measures (Lean et al., 2018). In cases where this is not a successful strategy, medications such as metformin can be taken regularly, or insulin injections administered.

Despite extensive research and knowledge of effective approaches for controlling/preventing T2D, new cases of T2D continue to rise rapidly and in 2021 was the cause of 6.7 million deaths (Sun et al., 2022), remaining one of the leading causes of death worldwide. The mechanistic cause of T2D at a cell biology level is still poorly understood, so further research is required in order to more effectively understand, prevent and treat type 2 diabetes.

1.1.1 Glucose homeostasis

Glucose homeostasis is essential to maintaining healthy tissue. Glucose levels are balanced by two hormones - glucagon and insulin. When there is an energy deficit, glucagon responds to low glucose levels by increasing gluconeogenesis and glucose release from the liver, along with breaking down glycogen stores in the body. These glycogen stores are replenished from dietary glucose.

Elevated plasma glucose levels are detected by pancreatic beta cells, which respond by secreting insulin, as shown in **Figure 1-1**. This insulin release is fast-acting; consuming 75 g of glucose will cause plasma insulin to rise 10-fold in 30 minutes (Fu et al., 2013). Insulin works in a number of different ways to lower plasma glucose levels, one of which is through stimulating the uptake of glucose into fat and muscle cells via the glucose transporter GLUT4.

One of the defining aspects of type 2 diabetes is insulin resistance, in which cells require larger amounts of insulin in order to have an effect. While the mechanisms behind insulin resistance are complex, dysregulation of GLUT4 is one of the contributing factors (Kahn, 1992). For example, GLUT4 heterozygous knockout mice have been shown to develop insulin resistance and diabetes, and GLUT4 expression is decreased in patients with type 2 diabetes (Stenbit et al., 1997).

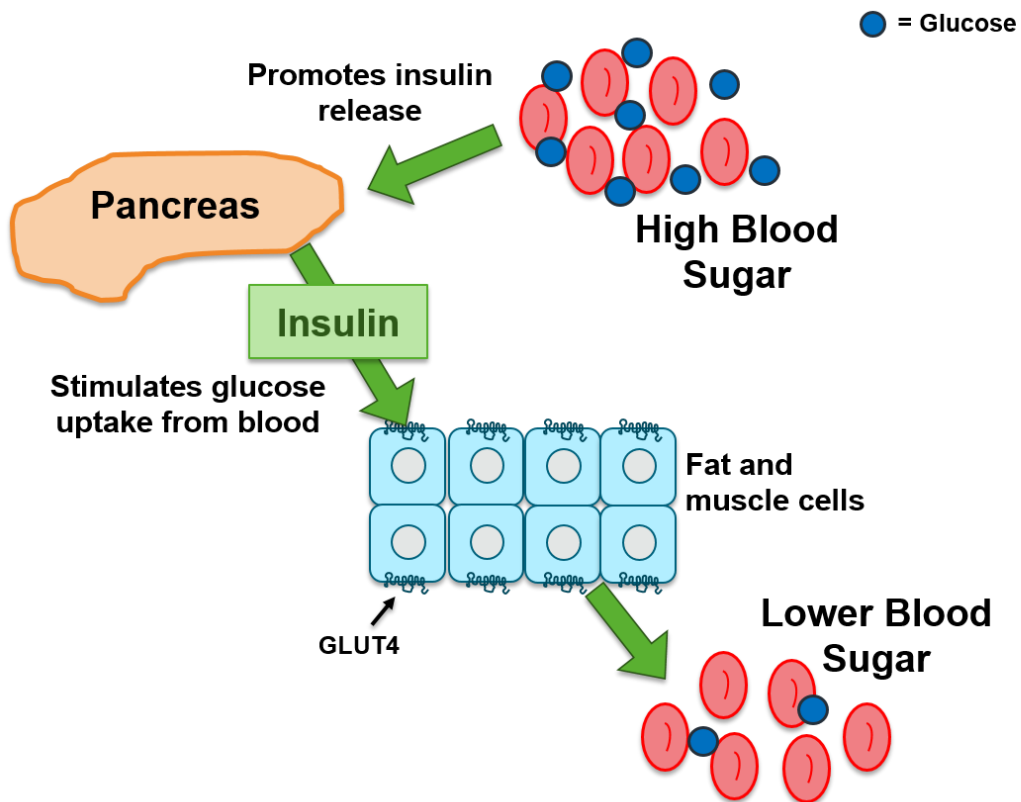


Figure 1-1 – Insulin drives glucose uptake into fat and muscle cells

Elevated blood glucose is detected by the pancreas which then releases insulin. Amongst other things, insulin acts to stimulate glucose uptake from the blood into fat and muscle cells through the action of Glucose Transporter Type 4 (GLUT4)

1.2 GLUT4 trafficking

1.2.1 Membrane trafficking

The secretory and endosomal networks are a series of membrane-bound compartments containing receptors and enzymes which enable the processing and transport of cargo proteins, including membrane proteins. Membrane proteins make up around 25% of a cell's total proteome, and have a broad range of functions in maintaining cellular health, such as cell signalling, nutrient transport, cell adhesion and cell migration (Uhlen et al., 2015). Proteins undergo their initial synthesis, folding and processing steps in the interconnected flattened membrane structure called the endoplasmic reticulum (ER). They are then transported to the Golgi apparatus, a series of membrane-bound stacked cisternae, to undergo further processing before being transported to the cell membrane via intracellular vesicles in a process called exocytosis. Once at the cell membrane, proteins are internalised via endocytosis, and then have one of four different fates (**Figure 1-2**). The first route is transport to the lysosome via the late endosome for degradation (Cullen and Steinberg, 2018). Proteins can be saved from the degradative fate to be recycled through the endosomal system and back to the plasma membrane, either directly from the early endosome in a fast recycling loop (the second route), or via the recycling endosome in a slow recycling loop (the third route) (Grant and Donaldson, 2009). Finally, in the fourth route, a protein can undergo retrograde trafficking back to secretory compartments such as the trans-Golgi network (TGN), Golgi and the ER (Johannes and Popoff, 2008), where it re-enters the secretory pathways and may undergo further modifications. These different routes are mediated by a vast range of different adaptors, scaffold proteins and enzymes.

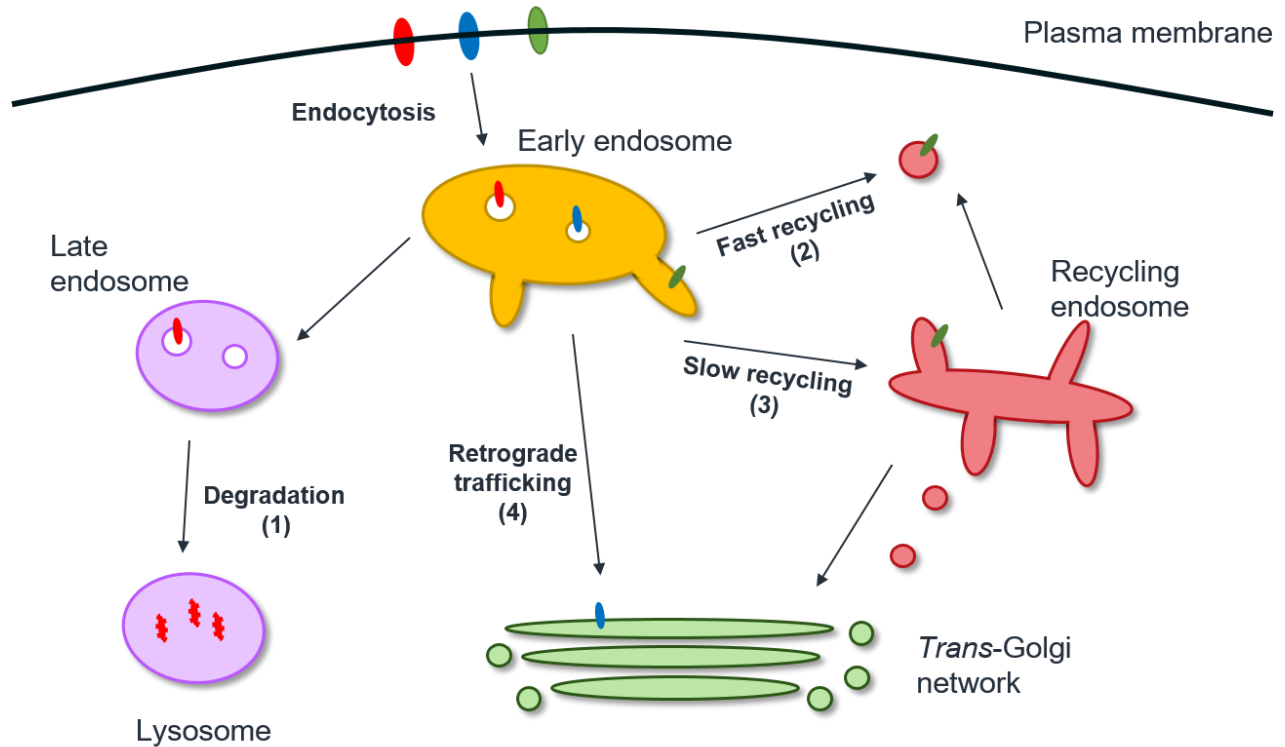


Figure 1-2 - Pathways in the endosomal network

Following endocytosis, membrane proteins have 4 different fates: 1) degradation through transport to the late endosome and lysosome (represented by the red circle) 2) fast recycling from the early endosome directly to the plasma membrane 3) slow recycling to the plasma membrane through the recycling endosome (represented by the green circle) 4) retrograde trafficking to the secretory pathway (TGN, Golgi or ER) (represented by the blue circle)

1.2.2 GLUT4 trafficking itinerary

Early research into the insulin-stimulated glucose uptake into adipocytes and muscle cells identified a unique, insulin-regulatable glucose transporter called GLUT4 (James et al., 1988; James et al., 1989). This 12-membrane-spanning protein is primarily expressed in adipocytes and muscle cells, and is released from an intracellular pool to the cell surface in the presence of insulin (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Watanabe et al., 1984).

In basal conditions, GLUT4 is effectively excluded from the plasma membrane, with as little as 1% of GLUT4 present at the cell surface in brown adipocyte tissue (Slot et al., 1991b). Insulin action redistributes GLUT4 localisation to the cell surface, with a corresponding increase in glucose uptake (James et al., 1986; Slot et al., 1991b). The mechanisms behind this redistribution have been the focus of intensive research and the subject of multiple reviews (Bryant et al., 2002; Leto and Saltiel, 2012).

GLUT4 constantly recycles through endosomal compartments in a retrograde trafficking route which includes the TGN, endosomes, vesicles and the plasma membrane (Jhun et al., 1992). These GLUT4-containing structures are collectively termed the GLUT4 storage compartment (GSC). Low levels of surface GLUT4 are partially accounted for by its unusually low exocytosis rate in basal conditions, which is increased through insulin action (Jhun et al., 1992; Karylowski et al., 2004; Satoh et al., 1993; Yang and Holman, 1993). While GLUT4 partially co-localises with other membrane proteins such as the constitutively recycling transferrin receptor (TfR), GLUT4 exocytosis is 10-fold slower than TfR in the absence of insulin, showing that there are aspects of GLUT4 trafficking which are separate from the general routes (Holman et al., 1994; Jhun et al., 1992; Tanner and Lienhard, 1987).

A distinct pool of highly GLUT4-enriched vesicles was identified which are rapidly mobilised to the cell surface in the presence of insulin, known as insulin responsive vesicles (IRVs) (Hashiramoto and James, 2000; Kandrор and Pilch, 1996; Martin et al., 1996). Along with GLUT4, a major component of these IRVs is the insulin-responsive aminopeptidase (IRAP) protein, which follows the same trafficking itinerary as GLUT4, and exhibits similar endo- and exocytosis rates (Garza and Birnbaum, 2000; Kandrор et al., 1994; Ross et al., 1997). Although not well characterised, IRAP may play a role in the formation of these IRVs (Shi et al., 2008).

A two-step model was proposed to explain GLUT4's intracellular sequestration and insulin-stimulated release, which incorporates both the general insulin-stimulated redistribution of membrane proteins, and the specific release of GLUT4 from a storage pool (Coster et al., 2004). In basal conditions, a proportion of GLUT4 constitutively recycles through the endosomal system to the cell surface in a fast recycling loop (**Figure 1-3**). However, a large proportion of GLUT4 is concentrated in a slow recycling loop between the IRVs, the recycling endosome and the TGN, acting as a storage pool keeping GLUT4 away from the cell surface (Govers et al., 2004; Livingstone et al., 1996). Insulin acts to increase the exocytosis rates across the GLUT4 storage compartment, as well as specifically stimulating the GLUT4-enriched IRVs to transport GLUT4 to the cell surface (**Figure 1-3**) (Coster et al., 2004; Kanai et al., 1993; Yang and Holman, 1993). This accounts for the much larger increase in surface GLUT4 compared to other membrane proteins in the presence of insulin.

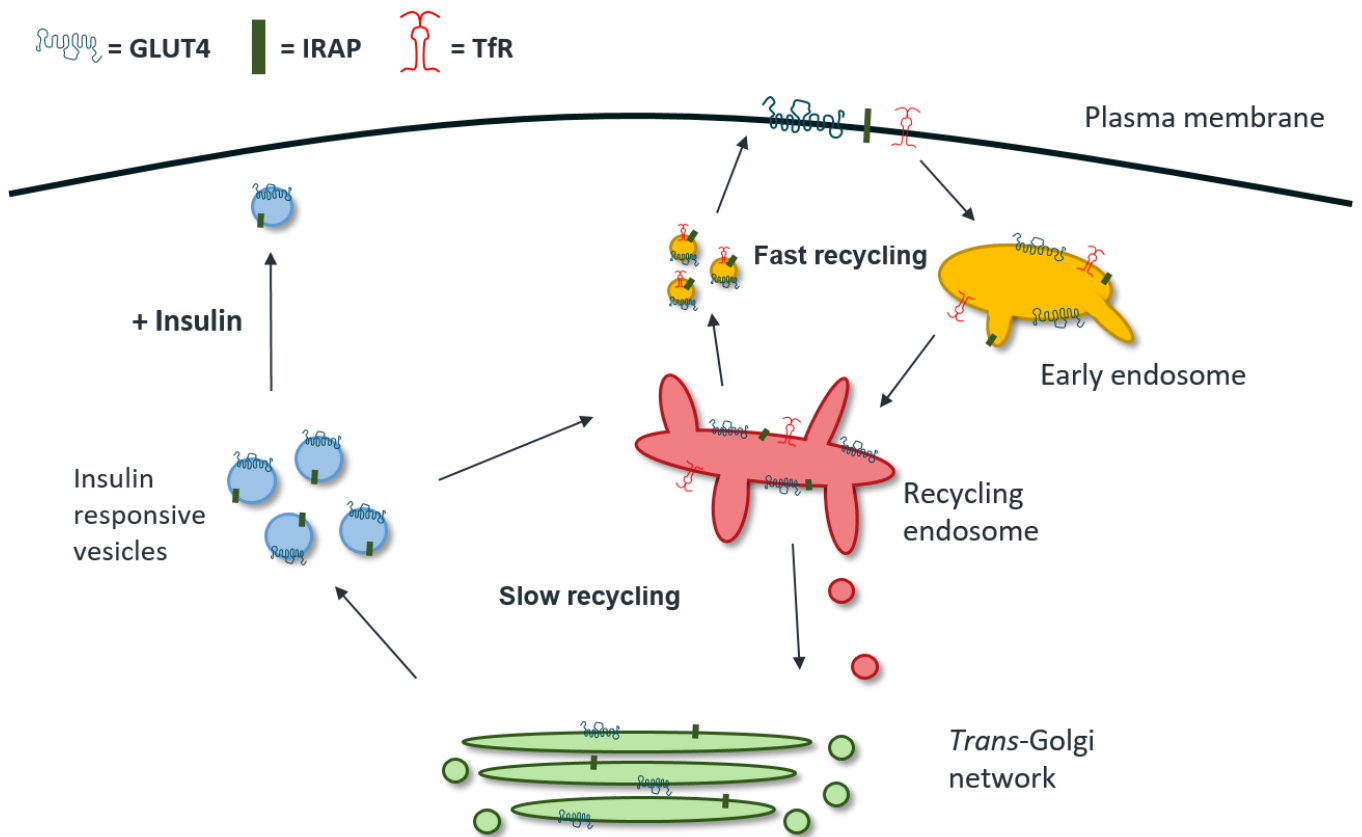


Figure 1-3 - GLUT4 trafficking itinerary

In basal conditions, GLUT4 has two transport loops. A small proportion of GLUT4 is constitutively transported to the cell surface through a fast recycling loop. The majority of GLUT4 is kept away from the cell surface through dynamic recycling between IRVs and the TGN. Insulin stimulates the translocation of GLUT4 to the cell surface through mobilising the IRVs and through increasing exocytosis rates.

1.3 GLUT4 internalisation

1.3.1 Endocytosis

Membrane proteins are internalised through a process called endocytosis, whereby membranes are folded into membrane pits or invaginations which pinch off to form intracellular vesicles. The best characterised method is clathrin-mediated endocytosis (CME), with other endocytosis mechanisms often being collectively referred to as “Clathrin-independent endocytosis” (CIE). In reality, CIE represents a range of different pathways that have distinct components and characteristics (Mayor et al., 2014). Three methods of endocytosis are summarised below and in **Figure 1-4**.

Clathrin is a molecular scaffold containing light and heavy chains, which together form clathrin ‘triskelion’ structures. (Kirchhausen and Harrison, 1981). These interact to make a basket-like lattice over the membrane, forming a coat which facilitates the internalisation of cargo through the recruitment of further scaffold and adaptor proteins (Kaksonen and Roux, 2018). The clustering of these proteins causes membrane bending until it forms a ‘clathrin coated pit’ which then undergoes constriction and scission through the action of BAR domain proteins and the GTPase dynamin (Kirchhausen et al., 2014). This results in a vesicle which is separated from the membrane. The clathrin coat and adaptors then ‘uncoat’ from the vesicle, which is then transported to the early endosome.

Clathrin independent mechanisms of endocytosis have been less well characterised, but act at areas of the membrane enriched in cholesterol, glycosphingolipids and Glycosylphosphatidylinositol (GPI)-anchored membrane proteins, termed lipid rafts. These lipid rafts can form caveolae (small invaginations in the plasma membrane) through the

polymerisation of cholesterol-binding caveolin proteins (Okamoto et al., 1998; Rothberg et al., 1992). Although these caveolae structures are generally relatively immobile, many proteins have been shown to be internalised via caveolae in a ligand specific manner (Benlimame et al., 1998; Parton et al., 1994; Schnitzer et al., 1994). This process also requires the GTPase dynamin for vesicle scission (Pelkmans et al., 2002).

Clathrin- and caveolae- independent tubular structures budding directly from the plasma membrane have also been identified (Kirkham et al., 2005; Llorente et al., 1998). These have been termed CLathrin-Independent Carriers (CLICs). The mechanisms which drive the formation of these structures were originally unclear due to the absence of any coat structures. This was explained by a model involving the carbohydrate-binding protein galectin-3, in which galectin-3 drives membrane bending and entry into endocytic pits (discussed in more detail in section 1.6) (Lakshminarayan et al., 2014). While some CLICs are generated independently of galectin-3, there is some evidence that these may be formed through the action of other galectins, such as galectin-4 (Lakshminarayan et al., 2014).

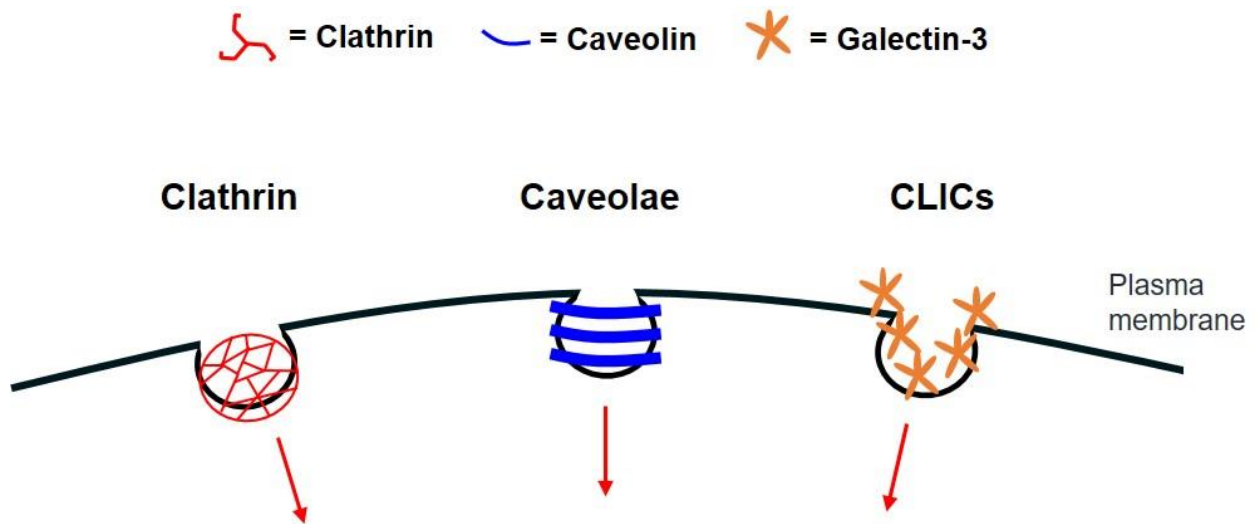


Figure 1-4 - Methods of endocytosis

Three examples of endocytosis mechanisms. The first involves clathrin, which forms a basket like lattice due to its triskelion structure, coating membrane buds. Caveolae-mediated endocytosis involves caveolin proteins which act as stabilising proteins to membrane invaginations. The non-coated clathrin-independent carriers (CLICs) act in a dynamin-independent manner, and have been shown to form through the binding of galectin-3 at the cell surface.

1.3.2 GLUT4 endocytosis

GLUT4 endocytosis has been shown to occur via two different mechanisms. When cells are insulin-stimulated, GLUT4 is internalised solely via CME (Blot and McGraw, 2006; Nishimura et al., 1993). Evidence for this includes the presence of GLUT4 in clathrin-coated pits (Slot et al., 1991a; Slot et al., 1991b), and the observation that potassium depletion increases the amount of cell surface GLUT4 (Nishimura et al., 1993). Potassium is important for the formation of clathrin-coated pits, indicating that GLUT4 is internalised in a clathrin-mediated manner (Hansen et al., 1993; Larkin et al., 1986). In addition, GLUT4 internalisation in insulin-stimulated cells is dependent on its F⁵QQI amino acid motif and the AP2 clathrin adaptor, which are both implicated in CME (Blot and McGraw, 2006).

In basal conditions, only 20% of GLUT4 is internalised via CME, and instead primarily undergoes cholesterol-dependent endocytosis (Blot and McGraw, 2006; Ros-Baro et al., 2001). This is thought to be a more efficient mechanism of endocytosis for GLUT4, as the F⁵QQI motif used in CME has lower endocytosis efficiency compared to the more common YQQI motif (Blot and McGraw, 2006). This suggests that insulin stimulation switches GLUT4 endocytosis into a less efficient mechanism, accounting for the decrease in endocytosis observed with insulin stimulation (Jhun et al., 1992; Yang and Holman, 1993).

Although the GTPase dynamin has been shown to be important, the exact clathrin-independent mechanism for GLUT4 internalisation is not well understood (Al-Hasani et al., 1998). In particular, there has been some controversy over the involvement of caveolae in GLUT4 trafficking. Some studies have reported a co-localisation of GLUT4 with caveolae (Gustavsson et al., 1996; Karlsson et al., 2002; Scherer et al., 1994; Yuan et al., 2007). In addition, cells expressing a non-functional caveolin-1 mutant demonstrated an

accumulation of GLUT4 on the plasma membrane in the absence of insulin (Shigematsu et al., 2003).

In contrast, there have also been studies which do not find caveolin in GLUT4-enriched vesicles, nor does intracellular caveolin form complexes with GLUT4 (Kandror et al., 1995; Parton et al., 2002; Robinson et al., 1992). The authors in Kandror et al (1995) argue that the previous contrasting data had no evidence of the efficiency of their assay, and suggest that the data is a result of low sensitivity.

One suggestion for reconciling the conflicting data is that GLUT4 is not concentrated in individual caveolae structures, but is loosely associated around larger caveolae domains (Shigematsu et al., 2003). However, there has been no evidence for this, and the mechanisms behind the clathrin-independent endocytosis of GLUT4 remain unclear.

1.4 Models for studying GLUT4 trafficking

Good model systems are required for studying GLUT4's trafficking itinerary and its regulation. GLUT4 is physiologically primarily expressed in adipocytes and muscle cells and early GLUT4 research used primary rat adipose or muscle cells (Cushman and Wardzala, 1980; Holman et al., 1994; Watanabe et al., 1984). The murine 3T3-L1 fibroblast cell line which can be differentiated into adipocytes was then developed which allowed for easier cell culture. Although these models provided key information as to the localisation and insulin-regulated trafficking of GLUT4, key differences are observed in the GLUT4 trafficking of murine cell models compared with human cell models. For example, the magnitude of the insulin-stimulated increase of cell surface GLUT4 is 10-20 fold greater in murine cell models than human adipocytes (Bryant and Gould, 2020). In addition, the CHC22 clathrin which plays a key role in the formation of the GLUT4 storage compartment (GSC) in human cells is not found in murine cells. (Camus et al., 2020). This highlights the need to study GLUT4 trafficking in human cell models in conjunction with the established murine cell models.

GLUT4 has been shown to display insulin responsive trafficking in a range of cell lines where it is not endogenously expressed. This suggests that the mechanisms involved in insulin responsive trafficking are common across cells, which is consistent with the presence of key GLUT4 sorting proteins such as IRAP and sortilin across cell types. HeLa cell lines which stably express tagged versions of GLUT4 (such as HA-GLUT4-GFP) have been shown to demonstrate this insulin-dependent GLUT4 trafficking with a similar magnitude of response to human adipocytes (Camus et al., 2020; Haga et al., 2011; Morris et al., 2020). The kinetics are also comparable to 3T3-L1 adipocytes, despite the 10-fold difference in magnitude

(Morris et al., 2020). In addition, HeLa cells have similar levels of CHC22 as human muscle cells, where it is primarily expressed in the body (Esk et al., 2010). A key feature of insulin-responsive cells is the GSC, of which the syntaxin-6 protein is considered a marker in rodent cells (Foley and Klip, 2014; Shewan et al., 2003). A GSC which co-localised with syntaxin-6 was also observed in HA-GLUT4-GFP HeLa cells, sharing similar attributes to adipocytes and muscle cells (Camus et al., 2020).

In addition to attributes which make HeLa cells a good comparable model to human adipocyte or muscle cells, they also offer experimental benefits over other cell lines due to their versatility. 3T3-L1 fibroblasts and mesenchymal stem cells (MSCs) can both be differentiated into adipocyte-like cells but are only differentiated for a short amount of time, limiting the scope of longer term experiments. Moreover, they are resistant to genetic manipulation through transient transfection, as transfection requires sub-confluent cell cultures, whereas differentiation requires over-confluence. Large scale cell culture growth is also limited by the lack of suspension adipocyte cell lines. In contrast, despite the high degree of genetic variation (Liu et al., 2019), HeLa cells are compliant to transfection and can easily be grown in large quantities.

In summary, HA-GLUT4-GFP HeLa cell offers a good cell model for the study of GLUT4 trafficking which can be subjected to a wide range of molecular biology techniques. This allows for consistency of cell models across experiments, allowing the findings to be linked into one model. Using the tagged version of GLUT4 also provides an easy way to visualise and quantify surface GLUT4 as a proportion of total GLUT4.

1.5 N-glycosylation of GLUT4

1.5.1 Post-translational modifications of GLUT4

While the general framework of GLUT4's trafficking itinerary and the roles of different amino acid motifs have been well established, the regulation of the different trafficking steps is still not well understood (Blot and McGraw, 2008). In particular, little is known about the role of post-translational modifications.

Following the process of DNA transcription and translation, polypeptides are folded into membrane proteins in the ER. Additional molecules are added to/removed from the protein as it travels through the ER, Golgi apparatus and trans-golgi network (TGN). These post-translational modifications (which can occur co-translationally) include glycosylation, phosphorylation, ubiquitination, palmitoylation, and SUMOylation. These modifications are often highly important to the stability and function of a protein.

Ubiquitination is one post-translational modification in which the small ubiquitin protein is added to a lysine residue on a protein by a system of enzymes. Although there has not been extensive research into the role of ubiquitination in GLUT4 trafficking, ubiquitinated GLUT4 has been shown to be rapidly deubiquitinated to save it from the degradative fate (Lamb et al., 2010). Ubiquitination is also key to the insulin response of GLUT4, as deubiquitinated GLUT4 is prevented from entering IRVs (Lamb et al., 2010).

In regards to glycosylation, GLUT4 has a single *N*-glycan on its first extracellular loop (Ing et al., 1996) (**Figure 1-5**). Its exact role in GLUT4 function is not well understood, but there have been indications it plays a role in regulating GLUT4 trafficking (discussed in more depth in section 1.5.4). Understanding the structure and function of the *N*-glycan may contribute

to the overall understanding of how GLUT4 regulates whole body glucose homeostasis. Due to the presence on the *N*-glycan at the extracellular surface, it could also represent a key therapeutic target for the treatment of type 2 diabetes.

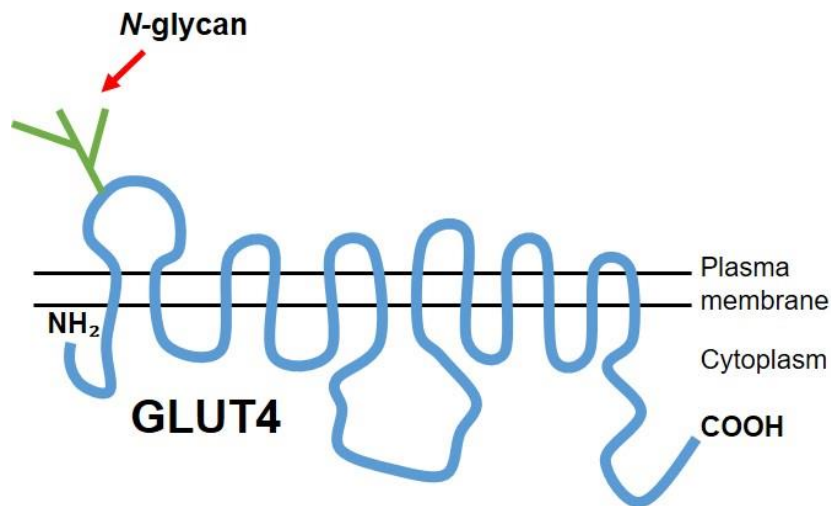


Figure 1-5 - GLUT4 has a single large N-glycan

GLUT4 is a 12-membrane spanning protein which has a single large N-glycan on its first extracellular loop. Although there has been no direct evidence, the size of the N-glycan makes the presence of a polymer such as poly(lactosamine) likely.

1.5.2 *N*-glycosylation

Glycosylation, the most common post-translational modification, involves the synthesis of an oligosaccharide chain onto a specific site of a protein (Apweiler et al., 1999). There are two main forms of glycosylation – *N*-glycosylation and O-glycosylation. *N*-glycosylation refers to oligosaccharides which are covalently attached to an asparagine residue by an *N*-glycosidic bond, while O-glycosylation refers to oligosaccharides attached to serine or threonine residues through a hydroxyl group (Brockhausen and Stanley, 2015; Stanley et al., 2015). O-glycans have not been reported on GLUT4, so the remainder of this section will focus on *N*-glycosylation.

N-glycans have a wide range of functions in the body. These include direct and indirect roles in glycoprotein folding and stability (Ferris et al., 2014), cell-cell recognition and adhesion (Gu et al., 2012), and immune recognition (Rudd et al., 2001). *N*-glycosylation is a challenging process to study, as unlike protein synthesis (which is encoded by DNA), it is not driven by a template. It is instead dependent on the availability of enzymes and monosaccharides, and the accessibility of the *N*-glycan (influenced by protein structure). This means that micro-heterogeneity is often observed, whereby different *N*-glycan structures are seen on the same site across a pool of the same protein.

The initiation of *N*-glycosylation involves the transfer of an oligosaccharide chain to an asparagine on the target protein which is within the consensus sequence N-X-S/T (where X is not proline) (Gavel and von Heijne, 1990). The oligosaccharide is pre-synthesised at the ER membrane on a dolichyl pyrophosphate anchor and consists of 3 glucose (Glc), 9 mannose (Man) and 2 *N*-acetyl-glucosamine (GlcNAc) residues (Glc₃Man₉GlcNAc₂) (Burda and Aepli, 1999). The terminal α -1,2 linked glucose is recognised by the multimeric enzyme complex

oligosaccharyltransferase (OST), which then scans the nascent polypeptide of a target glycoprotein for the consensus sequence (N-X-S/T) and transfers the oligosaccharide (Schwarz and Aeby, 2011). The glycoprotein then passes through the ER, where the three glucose monomers are trimmed with glucosidases, and the first mannose is trimmed by ER mannosidase 1 (Bieberich, 2014). The glycoprotein then travels into the Golgi, which is a series of membrane bound cisternae containing glycosylation enzymes which further process the glycan (**Figure 1-6**) (Bieberich, 2014).

Following transport into the Golgi, the *N*-glycan has two main antennae stemming from the first mannose unit - the α -1,3 arm and the α -1,6 arm. Four mannose monomers are trimmed by mannosidase 1 (MAN1), which then allows MGAT1 to add a GlcNAc monomer onto the α -1,3 arm. Two mannose monomers on the α -1,6 arm are then trimmed by mannosidase 2 (MAN2), allowing MGAT2 to add a GlcNAc monomer to that branch, forming a complex glycan. Both branches can then have further modifications added to it, such as fucose (added to the first GlcNAc by Fucosyltransferase 8), galactose (by Galactosyltransferase), or sialic acid (by Sialyltransferase), as well as additional branches (**Figure 1-6**).

A key modification is the addition of Gal(β 1-4)-GlcNAc(β 1-3) dimers (N-acetyllactosamine), through the alternative action of β 1-4 galactosyltransferases and β 1-3-*N*-acetylglucosaminyltransferases. This modification has a preference for the α 1,6 branch in multi-antennary *N*-glycans. The repeated addition of these dimers forms poly-*N*-acetyllactosamine (also called polylactosamine). This polymer can be in the form of one linear chain, or can form a branched structure through the addition of GlcNAc to internal galactose residues by β 1-6-*N*-acetylglucosaminyltransferases. Polylactosamine can act as a binding ligand for proteins such as the galectin family (described in more detail in 1.6).

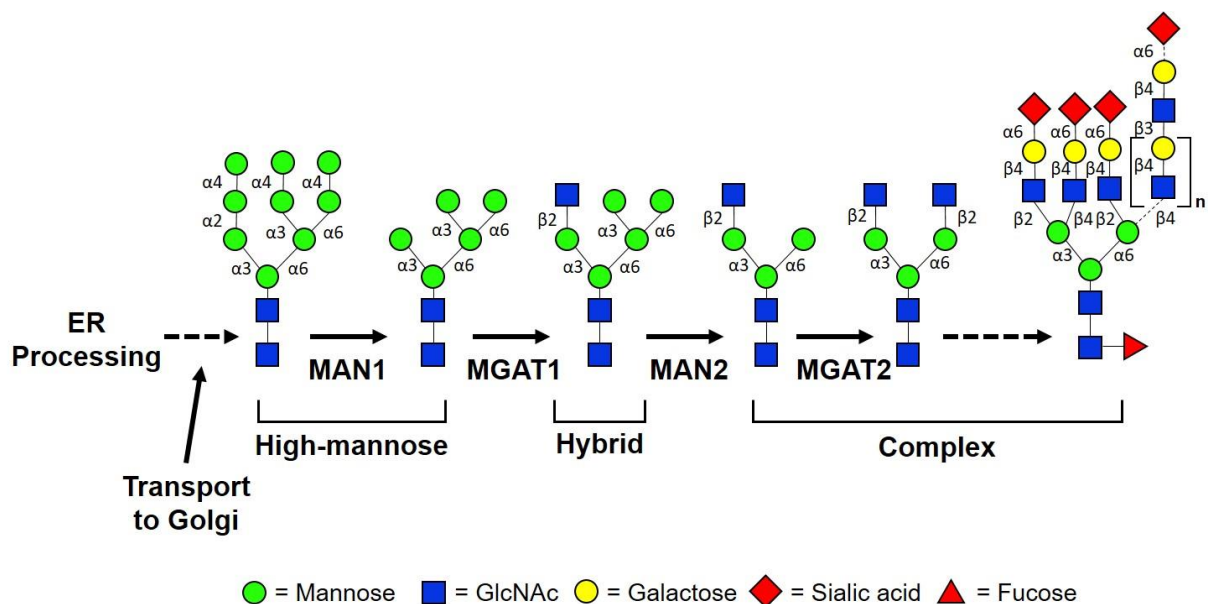


Figure 1-6 N-glycan synthesis in the Golgi

Following the initial transfer of an N-glycan to the protein and subsequent trimming in the ER, the protein is transported to the Golgi carrying a $\text{Man}_9\text{GlcNAc}_2$ N-glycan. Trimming of 4 mannose units by mannosidase I (MAN1) is followed by the addition of a GlcNAc unit onto the $\alpha 3$ branch by MGAT1. Mannosidase II (MAN2) then trims a further 2 mannose units from the $\alpha 6$ branch, allowing MGAT2 to add a GlcNAc unit, forming a complex glycan which can undergo further processing to add additional monosaccharide units.

Due to the non-templated nature of glycan synthesis, the final structure for each N-glycan depends on the availability of the enzymes present and competition between enzymes due to limited processing time available in each Golgi cisternae. Along with the complex structure previously described, N-glycan structures can also fall into two further categories which have not undergone all the processing steps to achieve a complex N-glycan (**Figure 1-7**). High mannose N-glycans are mannose-terminating structures, consisting solely of the core structure and non-substituted mannose. Hybrid N-glycans are structures which have one high-mannose branch and one complex branch. Different structures have different

specificities for glycan-binding proteins (lectins) and can affect the binding to a protein receptor, so the specific glycan structures presented on a glycoprotein can be key to its function.

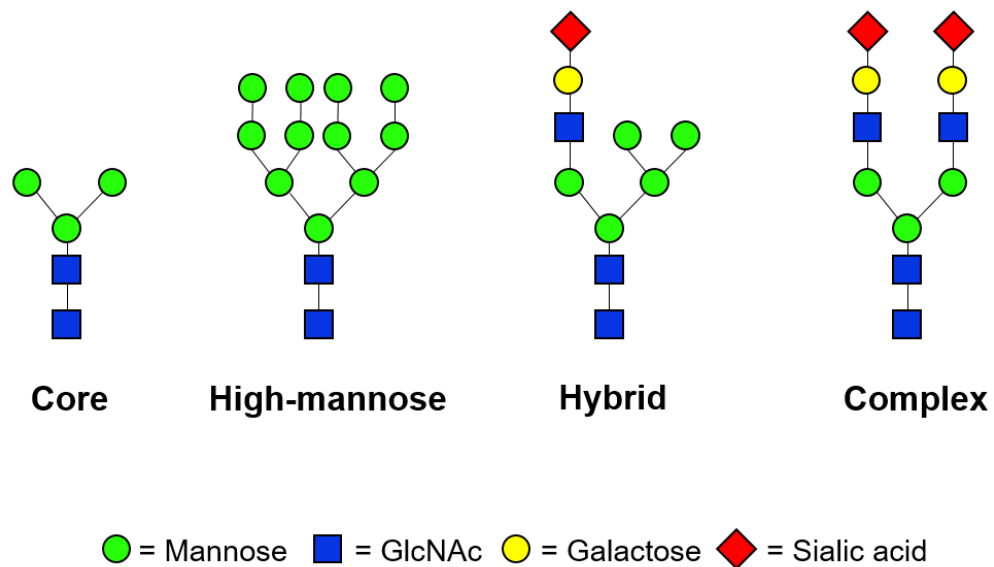


Figure 1-7 - Types of N-glycan

All N-glycan structures contain a $\text{Man}_3\text{GlcNAc}_2$ core structure. Structures can fall into one of three different categories: 1) High-mannose – consisting of GlcNAc and mannose only 2) Hybrid structures with aspects of both high-mannose and complex structure 3) Complex structures, which have both core branches modified with a minimum of one N-acetylglucosamine unit

1.5.3 Methods for studying *N*-glycosylation function

Understanding the role of glycosylation for a specific protein requires the right tools. Some studies have relied on a point mutation of the glycosylation site to remove its *N*-glycan (Ing et al., 1996; Zaarour et al., 2012). This offers some benefits over global treatments, as any effects observed can be attributed directly to that *N*-glycan, rather than introducing the possibility that effects are down to changes in interacting proteins. However, *N*-glycans often play crucial roles in protein folding and stability, in particular as a central part of the ER's folding and quality control machinery (Roth et al., 2010; Shental-Bechor and Levy, 2008). Mutation of the glycosylation site may therefore cause a reduction in protein expression and obscure any other functions the *N*-glycan has.

Using inhibitors of glycosylation enzymes removes the danger of affecting protein stability and folding, as these enzymes act post ER folding. For example, kifunensine is an inhibitor of mannosidase I, preventing the downstream processing in the Golgi and resulting in a GlcNAc₂Man₉ structure. Swainsonine inhibits mannosidase II, blocking the transition of an *N*-glycan from a hybrid to a complex form. The global nature of these inhibitors means that care is needed when interpreting results, as observations may be due to the altered *N*-glycan structure of binding partners rather than the specific protein of interest. However, using these inhibitors provides a more nuanced approach to altering glycan structure compared to the complete removal of the *N*-glycan, as the effect of smaller structural changes can be observed. This can also be achieved through siRNA-mediated knockdown or CRISPR-Cas9 mediated knockout of specific glycosylation enzymes.

1.5.4 GLUT4's *N*-glycan function in GLUT4 trafficking

While there is little known about GLUT4's *N*-glycan, a small number of studies have investigated a possible role in GLUT4 trafficking. The first study to attempt this was published in 1996, in which the glycosylation site of GLUT4, Asn57, was mutated in rat adipocyte cells, in order to compare the difference between surface HA-GLUT4 levels and surface HA-GLUT4(N57Q) (Ing et al., 1996). This data showed that HA-GLUT4 was able to respond to insulin as normal, but HA-GLUT4(N57Q) had no insulin response, which would suggest that the *N*-glycan plays a key role in GLUT4's ability to translocate to the cell surface in response to insulin. However, the authors added the caveat that expression levels of HA-GLUT4(N57Q) were so low that the experiment may not have been sensitive enough to pick up any changes. This is likely due to the role of the *N*-glycan in ER quality control and folding, which is a widely known role of *N*-glycosylation (Shental-Bechor and Levy, 2008).

Despite this, more recent studies also looked at the effect of mutating GLUT4's *N*-glycosylation site. Notably, two studies were released within a close time-frame which both looked at Wild-Type (WT) vs N57Q GLUT4 and came to opposite conclusions (Haga et al., 2011; Zaarour et al., 2012).

Haga *et al* used biotinylation assays to measure surface GLUT4 levels in HeLa cells. In basal conditions, there was an increased amount of surface GLUT4(N57Q) compared to WT, and a loss of insulin response in N57Q. This was also observed for kifunensine treated WT GLUT4 cells, which removes the bulk of the *N*-glycan through the inhibition of mannosidase I (Haga et al., 2011).

In addition, visualisation of HA-GLUT4-GFP in HeLa cells, 3T3-L1 adipocytes and L6 myocytes

by confocal microscopy demonstrated that GLUT4(N57Q) is observed on the cell surface in basal conditions, and WT GLUT4 is not (Haga et al., 2011). The insulin-stimulated increase in surface GLUT4 is also abolished for GLUT4(N57Q), but this may partly be due to GLUT4(N57Q) already being present at the cell surface.

While Zaarour *et al* agreed on the importance of GLUT4's *N*-glycan to its stability, they observed no difference in insulin response between HA-GLUT4 and HA-GLUT4(N57Q) when surface GLUT4 levels were measured using a fluorescent anti-HA antibody in a range of cell types (3T3-L1 pre-adipocytes, 3T3-L1 adipocytes, L6 myoblasts and HeLa cells). However, they did observe elevated basal levels of surface GLUT4(N57Q) compared to WT in HeLa cells (Zaarour et al., 2012). The findings from both studies suggest that GLUT4's *N*-glycan plays a role in regulating basal levels of GLUT4, although this conclusion is overlooked by the authors.

To try and reconcile the contrasting conclusions from these prior studies, Hirayama *et al* used novel fluorogenic probes to perform spatio-temporal analysis of GLUT4, using kifunensine to alter *N*-glycan structure (Hirayama et al., 2016). Live cell imaging using both cell-permeable and -impermeable probes visualised insulin-dependent GLUT4 trafficking to the cell surface. Following kifunensine treatment, GLUT4 still demonstrated insulin-stimulated trafficking to the cell surface. However, it is only present very transiently before being endocytosed back into the cell. At any fixed time point, it may therefore appear that there has been no insulin response. This suggests that GLUT4's *N*-glycan plays a role in GLUT4 retention at the cell surface, in contrast to the role suggested in a previous study in which the *N*-glycan is needed for the insulin-stimulated surface translocation of GLUT4 (Haga et al., 2011)

In summary, although there is a general consensus for GLUT4's *N*-glycan having a function in GLUT4 trafficking, it remains unclear as to what this function is and the mechanisms through which it is mediated.

1.5.5 Structure of GLUT4's *N*-glycan

Little has been studied with respect to the structure of GLUT4's *N*-glycan. It has been shown that the *N*-glycan is large, based on the observed 20-30 kDa decrease in molecular weight of GLUT4 when the *N*-glycan is removed (Haga et al., 2011). This size indicates that a polymer must be present on the *N*-glycan, as the maximum size of an *N*-glycan chain without polymers is around 4 kDa. This is most likely to be in the form of polylactosamine, as the other polymer structure known to exist on *N*-glycans (polysialic acid) is mostly found in the nervous or immune systems (Colley et al., 2014).

One study which utilised a FRET based system to analyse the specific glycoforms of GLUT4 revealed a mixed population of GLUT4, with both sialylated and non-sialylated forms (Haga et al., 2012). Sialylated GLUT4 demonstrated slower internalisation kinetics to non-sialylated GLUT4, showing that aspects of the *N*-glycan's structure are key to GLUT4 function. This study only looked at labelled sialic acid, so did not provide any details on other components of the *N*-glycan.

While structural analysis of GLUT4's *N*-glycan has been limited, structural analysis of another glucose transporter (GLUT1) has been achieved. Unlike GLUT4, GLUT1 does not exhibit insulin-sensitive trafficking, and instead is ubiquitously transported to the cell surface. However, the proteins share high sequence homology and both have an *N*-glycan on their first extracellular loops. Using anion-exchange chromatography, two different *N*-glycan structures were identified – high-mannose and complex type (Endo et al., 1990). The complex-type structures contain a polylactosamine chain with an average size of around 16 *N*-acetylactosamine units (Endo et al., 1990). This may therefore indicate that the large size of GLUT4's *N*-glycan is also due to an extended polylactosamine chain.

1.6 Galectins

Galectins are a family of proteins able to bind β -galactoside sugars, in particular within glycans containing *N*-acetyllactosamine units. They are known to contribute to a variety of different cellular functions, such as immune responses, adhesion, autophagy and cellular homeostasis (Johannes et al., 2018). Galectins are characterised by a carbohydrate recognition domain (CRD) with an affinity for β -galactoside and most commonly bind to *N*- and O-glycans. They all have roles both intracellularly and extracellularly, and are secreted through a non-conventional pathway which has been poorly characterised but which doesn't follow more established pathways of secretion (Hughes, 1999). There are three classes of galectins: the prototype class which contain only one CRD, the tandem repeat class which contain two CRDs, and the chimera class which has one CRD and an extended non-lectin N-terminal domain – the only member of which is galectin-3. Each galectin has different affinities for different attributes of *N*-acetyllactosamine-containing *N*-glycans, such as degree of branching, number of repeats and different modifications (Hirabayashi et al., 2002). In general, affinity for *N*-acetyllactosamine tends to increase as the number of units in a polylactosamine chain increases, and affinity for branched polylactosamine is greater than the linear form (Hirabayashi et al., 2002).

1.6.1 Galectins can regulate cell surface glycoprotein expression

It has been shown *in vitro* that galectin-3, while existing as a monomer, is able to form oligomeric structures with cross-linking abilities, through its N-terminal domain (Ahmad et al., 2004; Nieminen et al., 2007). This crosslinking ability allows galectin-3 to form rafts or lattices with glycoproteins under physiological conditions (Nieminen et al., 2007). These interactions are thought to play a key role in the intracellular trafficking of a variety of cell surface proteins. The exact role that galectins play has been complex to uncover, as seemingly opposing functions of galectin-glycoprotein interactions have been identified.

A number of studies have presented evidence that galectin interactions with glycoproteins form a lattice which increases the cell surface residence time, slowing the rate of endocytosis. MGAT5 is an *N*-glycan processing enzyme which is key to the formation of polylactosamine, and cells lacking this enzyme exhibited a decrease in surface residence of cytokine receptors such as EGFR and T β RII (Partridge et al., 2004). Given that polylactosamine is a binding ligand for galectin-3, this suggests that galectin-3 may be involved in anchoring these proteins at the cell surface (Partridge et al., 2004). This was also observed for VEGF-R (Markowska et al., 2011). While this has been better characterised for galectin-3, there is some evidence that other galectins such as galectin-1 also have cross-linking abilities able to retain proteins at the cell surface (Belardi et al., 2012).

In contrast, galectin-3 can also mediate endocytosis, as observed for β 1 integrin in breast carcinoma cells (Furtak et al., 2001). Evidence was found for a model in which galectin-3 drives the formation of clathrin independent carriers (CLICS) (Lakshminarayan et al., 2014). This proposes that oligomeric galectin-3 structures interact with the glycoprotein cargo along with glycosphingolipids (GSLs), forming a cluster. This clustering generates enough

mechanical strain to create endocytic pits, through which the cargo is internalised. The model is called the 'GL-Lect hypothesis' (Johannes et al., 2016). While this study focused on galectin-3, the finding that some CLICs co-localised with galectin-4 instead of galectin-3 indicated that this function could be seen in multiple galectins (Lakshminarayan et al., 2014).

A study which reconciles these two functions proposes that action of galectins is dependent on the strength of the lattice, determined by galectin affinity and concentration. Glycan structure also contributes, as a branched polylactosamine structure provides more binding sites for galectins, leading to higher concentration (Mathew and Donaldson, 2018). This acts along a spectrum; galectin-3 binding initially stimulates the GL-Lect model whereby endocytosis is promoted. As galectin-3 concentration rises further, the lattice increases in size/strength and the glycoproteins are retained at the cell surface, as demonstrated in **Figure 1-8** (Mathew and Donaldson, 2019). This switching between modes of action can make studying galectins complex, as changing galectin amounts can switch the mode of action.

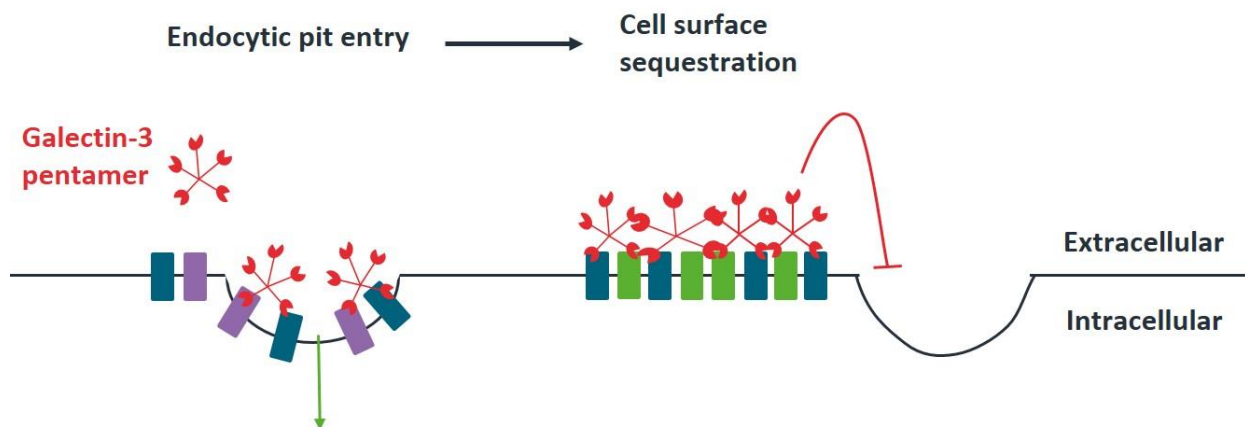


Figure 1-8 - Model for Galectin-3 action

The oligomerisation of galectin-3 into a pentameric structure allows it to form glycoprotein-galectin interactions which bend membranes into CLICs, facilitating the endocytosis of membrane proteins. When the concentration of galectins or glycan ligands increases, the galectin-glycoproteins form a lattice which blocks their entry into endocytic pits, causing retention at the cell surface. Adapted from Mathew and Donaldson, 2019.

1.6.2 Galectins and Type 2 diabetes

Due to their wide-ranging functions, galectins have been implicated in many diseases, including cancer, cardiovascular disease and autoimmune diseases (Gao et al., 2013; Girotti et al., 2020; Klyosov and Traber, 2012; van der Hoeven et al., 2016). Among these, T2D has also been shown to be associated with altered galectin levels.

Galectin-3 is expressed in adipocytes, and an association between galectin-3 and T2D and/or obesity has been found both in animal studies and from human plasma. For example, galectin-3 knockout studies in mice display increased adiposity, impaired glucose uptake and susceptibility to T2D (Darrow and Shohet, 2015; Pang et al., 2013; Pejnovic et al., 2013). In contrast, administration of additional galectin-3 in mice also displayed glucose intolerance and insulin resistance (Li et al., 2016)

In human studies, galectin-3 levels were elevated in human sera taken from individuals who were diagnosed with T2D compared to healthy individuals (Weigert et al., 2010; Yilmaz et al., 2015). A conflicting study demonstrated a correlation between lower serum galectin-3 concentration and insulin resistance (Ohkura et al., 2014). However, this study only looked at 20 patients, providing no negative controls of healthy individuals, so all patients may already have elevated galectin-3 levels compared to normal levels. In addition, the patients were all taking different diabetic medication, but this factor was not included in the analysis. Given that metformin treatment has been shown to be associated with lower galectin-3, this may be a major contributing factor which has been ignored (Weigert et al., 2010). The evidence therefore points to an increase in serum galectin-3 levels being associated with type 2 diabetes in humans, although this may differ in mice. Given the importance of galectin-3 concentration to its mode of action, it is also possible that both lowered and

increased galectin-3 serum levels may cause downstream effects resulting in type 2 diabetes (Mathew and Donaldson, 2018).

Galectin-1 has been shown to be secreted by 3T3-L1 adipocytes and upregulated in the blood plasma of diabetic patients (Liu et al., 2009; Wang et al., 2004). Galectin-1 is also elevated in the subcutaneous adipose tissue of patients with type 2 diabetes, where it significantly reduces glucose uptake (Fryk et al., 2016).

The mechanistic relationship between galectins and type 2 diabetes is not yet understood, but their association could indicate a role in regulating GLUT4 trafficking. In support of this, galectin-3 treatment has been shown to reduce insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (Li et al., 2016).

Another study investigated the effect of increasing galectin affinity for their glycan ligands. This was achieved by increasing *N*-glycan branching through GlcNAc addition (Hirabayashi et al., 2002; Lau et al., 2007). Increased *N*-glycan branching resulted in enhanced surface GLUT4 levels in HEK293T cells, suggesting that galectin binding affects GLUT4 trafficking. Further evidence is needed to define the action of galectins in normal conditions, as artificially increasing the *N*-glycan branching may be switching galectins into a different mode of action.

1.7 Project Aims

The overall aim of this project is to characterise the structure and function of GLUT4's *N*-glycan, in order to build a model for the way *N*-glycan structure interfaces with function to affect GLUT4 trafficking. This project will utilise the HA-GLUT4-GFP HeLa cell model system in order to have one consistent model throughout the whole project which retains insulin-responsive trafficking of GLUT4 and will facilitate a range of experimental procedures.

Chapter 3 explores the function of the *N*-glycan by altering the structure using glycosylation enzyme inhibitors. The HA and GFP tags will allow quantification of the proportion of GLUT4 at the cell surface in the presence of these inhibitors. The overall localisation of GLUT4 in the presence of glycosylation inhibitors will also be analysed by subcellular fractionation. GLUT4 glycosylation will also be tracked over time using a RUSH assay to test a hypothesis of sequential glycosylation.

In **chapter 4**, the structure of GLUT4's *N*-glycan will be investigated using monosaccharide composition analysis. A protocol for GLUT4 purification from HeLa cells using a GFP nanotrap will be developed, followed by the optimisation of fluorescently-linked reverse phase HPLC for monosaccharide analysis. The resulting peaks will be quantified in order to develop a structural model for GLUT4's *N*-glycan.

Finally, the aim of **chapter 5** is to bring together the function and structure into one model by exploring the role that galectins play in the function of GLUT4's *N*-glycan. This will involve using lactose as a competitive inhibitor for galectin binding to observe any effects on surface GLUT4 levels. Specific galectin candidates will then be selected to explore further through

specific inhibition/knockdown and through increasing their extracellular concentration using recombinant purified protein.

2 Methods

2.1 Mammalian cell culture

2.1.1 Adherent Cell culture

All adherent cell lines were cultured in DMEM (Gibco) containing 10% FBS (Gibco), 1% Glutamax (Gibco) and 1% Penicillin-Streptomycin (Gibco). Cells were kept at 37 °C in 5% CO₂ and generally grown in T75 flasks. Cells were passaged when confluent by washing with 10 mL 1X Dulbecco's phosphate-buffered saline (Gibco) and detachment with 2mL 1X trypsin-EDTA (Gibco). Cells were incubated for 5 minutes at 37 °C then resuspended in 8 mL cell medium for transfer into a fresh culture flask with a total of 12 mL cell medium. For plating into 6- or 24- well dishes, resuspended cells were centrifuged at 1000 rpm for 5 minutes, and resuspended in 1 mL. Cells were counted using a haemocytometer or the Vi-Cell XR automatic cell counter (Beckman Coulter), and desired numbers of cells transferred into the plates.

2.1.2 Adapting HeLa adherent cells to suspension cells

To adapt adherent cells for suspension, HeLa cells cultured were as normal in DMEM + 10% FBS. After two passages, % FBS was halved until cells are growing in 2.5% FBS. Cells numbers were grown to around 9×10^7 and then transferred to a suspension flask containing 50% DMEM+ 2.5% FBS, and 50% MEM media. Suspension cells were incubated on a shaker at 37 °C in 5% CO₂. Following 2 passages, cells were cultured in MEM (Joklik Modification) (Sigma) with 5% FBS, 1% Glutamax and 1% Penicillin-Streptomycin. For passaging, cells were

counted using the Vi-Cell XR automatic cell counter (Beckman Coulter). The desired volume of cell culture was then transferred into a fresh suspension flask.

2.1.3 Freezing of cells and revival

Adherent cells were grown to 80% confluency in T75 flasks, then washed in 10 mL 1X PBS and trypsinised with 2 mL trypsin (as in 2.1.1). Cells were then resuspended in 8 mL DMEM. Adherent or suspension cells were centrifuged at 1000 rpm for 5 minutes, and resuspended in 90% (v/v) FBS (Gibco), 10% (v/v) DMSO (Chemcruz). 1 mL aliquots transferred to cryovials and frozen in a Nalgene “Mr FrostyTM” Freezing container (Thermo) placed in a -80 °C freezer for at least 24 hours before transferring to liquid nitrogen storage.

Cells were revived by placing frozen vials in a 37 °C water bath until thawed, then transferring to a T75 flask containing 12 mL DMEM media (for adherent) or into a suspension flask with 30 mL MEM (Joklik modification) media.

2.1.4 Making up sugar solutions in growth media

200 mM sugar solutions (Lactose, sucrose, maltose, fructose, glucose, galactose, glucosamine or lactulose) were made up by dissolving in serum-free DMEM (Gibco).

Solutions were diluted 1:3 with dH₂O to equal osmolarity as serum-free DMEM, and 1 mM MgCl₂ and 1 mM CaCl₂ were added to mitigate any effects on cell adhesion and migration.

Solutions were filter-sterilised and added to cells at 37 °C.

2.1.5 Kifunensine or swainsonine treatment

Kifunensine or Swainsonine (Cayman Chemical) were added to cells at 2 µg/mL or 10 µg/mL respectively at time of plating. Cells were incubated for 48 – 72 hours before harvesting.

2.1.6 Inhibition of galectin-1 or -3 binding

Galectin-1 (GB1490) and Galectin-3 (GB1107) small molecule inhibitors were gifted from Galecto. 20 mM stock solutions were made up in DMSO. 10 µM of each inhibitor was added to HA-GLUT4-GFP HeLa cells for 15 minutes following serum starvation (described in following section)

2.1.7 Serum starvation of HA-GLUT4-GFP HeLa cells for flow cytometry

5 x 10⁵ HA-GLUT4-GFP HeLa cells were seeded in a 6-well dish the previous day. Cells were washed in serum-free DMEM (Gibco) and serum starved in serum-free DMEM for 2 hours. Following serum starvation and/or treatments, cells were fixed in 2% PFA, then washed 3x with ice cold 1X PBS and stored overnight at 4 °C.

For sugar experiments, 2 ml of each sugar solution (described in 2.1.4) was added in place of serum-free DMEM for the 2 hour serum starvation.

For galectin-1 and -3 assays, recombinant galectin (range between 0-1.5 µM or 0-3 µM respectively) was added to cells at the start of serum starvation. For galectin-1 and -3 inhibition, 10 µM small molecule inhibitors were added for 15 minutes following 2 hour serum starvation.

For insulin stimulation experiments, 1 µg/mL Insulin was added for 20 mins following 2 hours serum starvation.

2.1.8 Surface staining for flow cytometry

HA-GLUT4-GFP HeLa cells were blocked in ice cold 2% BSA in PBS for 45 minutes. They were then stained with primary antibody in 2% BSA in PBS for 1 hour. After this they were washed three times with 2% BSA and stained using goat anti-mouse Alexa 647 secondary antibody (1:500) for 1 hour. Cells were washed 3x in ice-cold 2% BSA, and were then lifted with a cell scraper in 1ml 2% BSA. The samples were collected into a 1.5ml Eppendorf, which were then centrifuged at 600 xg for 8 min at 4 °C. Supernatant was discarded and pellet was resuspended in 350 µL 2% BSA.

2.1.9 Transient transfection of DNA plasmids

Cells were seeded to 70% confluency in a 6-well dish. 2 µg sgRNA-Cas9 plasmid was mixed with serum free DMEM up to 200 µL, then 4 µL XtremeGENE™ HP DNA reagent (Sigma) was added (for 2:1 ratio). Mixture was incubated for 15 minutes, then added dropwise to HeLa cells in 2 mL DMEM. Lysates were collected 24-72 hours after transfection.

2.1.10 siRNA transfection of HA-GLUT4-GFP HeLa cells

On the day before transfection, HA-GLUT4-GFP HeLa cells were seeded in a 6-well plate at 1×10^5 cells per well in 2 ml of DMEM and incubated in under normal growth conditions. On day of transfection, 100 µL serum-free medium and 0.6 µL siRNA (stock siRNA = 20 µM) were mixed. 12 µL HiPerfect transfection reagent (Qiagen) was added and mixed, and samples were then incubated for 20 min at room temperature. After this, the medium was removed from the 6-well plate, and 100 µL of the siRNA mix was added dropwise to the cells and gently swirled. 2.2 mL complete medium was then added, and the cells were then incubated under normal growth conditions for 72 hours.

2.1.11 CRISPR Cas9 transfection into HeLa cells for Knock-out

Cells were seeded to 70% confluency in a 6-well dish. 2 µg sgRNA-Cas9 plasmid was mixed with serum free DMEM up to 200 µL, then 4 µL XtremeGENE™ HP DNA reagent (Sigma) was added (for 2:1 ratio). Mixture was incubated for 15 minutes, then added dropwise to HeLa cells in 2 mL DMEM. After 48 hours, 5 µg/mL puromycin (Gibco) was added to cells and incubated for 72 hours before refreshing media. Cells were trypsinised and diluted 25x in DMEM, and plated into 5x 10cm² to allow distinct colonies to grow from single cells. Colonies were then collected using sterile discs soaked in trypsin, and transferred into a 24 well plate. Once cells had grown off the disc, they were divided between a 6 well dish for validation by western blot, and T75 dish for continuing cell culture.

2.1.12 CRISPR Knock-in

DNA transfection was performed as described in 2.1.11, with 10 µM ssODN repair template combined with 500 ng of two 2 gRNAs flanking either side of the knock-in site.

2.1.13 PNGase treatment

Confluent HA-GLUT4-GFP HeLa cells were lysed in 200 µL 1x sample buffer (5X sample buffer described in section 2.6.1, diluted 1:5 with 1X PBS) using a cell scraper. 20 µL of lysate was combined with 10 µL glycoprotein denaturing buffer (5% SDS, 400 mM DTT), and dH₂O was added up to 100 µL. Proteins were denatured by heating reaction at 100 °C for 10 minutes. Samples were chilled on ice and centrifuged for 10 seconds. 20 µL of 10X glycobuffer 2 (500 mM sodium phosphate pH 7.5), 20 µL 10% NP-40 and 60 µL H₂O were then added to the samples, followed by 10 µL PNGase F. Samples were mixed gently and incubated at 37 °C.

2.2 Molecular biology techniques

2.2.1 Transformation of DNA plasmid into *E.coli* cells

2 µL of DNA plasmid was mixed with 20 µL ice-cold chemically competent DH5α cells (NEB) (for DNA purification) and BL21 cells (NEB) (for protein purification). Mixture was incubated on ice for 30 mins, then heat shocked at 42 °C for 30s and returned to ice for 2 min. 300 µL SOC medium (NEB) was then added and cells were incubated in a shaker at 37 °C for 1 hour. Cells were plated onto LB agar plate containing 100 µg/mL AMP and incubated overnight at 37 °C.

2.2.2 DNA miniprep

A single colony was selected from *E.coli* transformation and used to inoculate 10 mL LB culture + ampicillin (AMP) (1:1000). Culture was then incubated overnight at 37 °C in a 200 RPM shaker, before centrifuging at 10,000 xg for 5 minutes. DNA purification was then performed using WizardPlus SV minipreps DNA purification system.

2.2.3 DNA midiprep

A single colony was selected from transformed *E.coli* plated onto an LB agar plate and used to inoculate 5 mL LB culture plus AMP (1:1000). Culture was then incubated overnight at 37 °C in a 200 RPM shaker. The 5 mL culture was then diluted in 300 mL LB medium + AMP (1:1000) and again incubated overnight at 37 °C in a 200 RPM shaker. Cells were then harvested by centrifugation at 5000 rpm for 15 minutes at 4 °C. DNA purification was then performed using Qiagen HiSpeed plasmid midi kit.

2.2.4 Protein purification from *E.coli* cells

A single colony of transformed BL21 cells was picked and grown in 10 mL LB medium + AMP (1:1000) overnight. 1 mL AMP and 1 mL overnight culture was added to 1L flask containing LB medium and incubated in shaker (200 rpm) at 37 °C until OD~0.5. 0.25mM IPTG was added to culture, which was then incubated overnight at 20 °C, 200rpm.

Culture was spun down in 750 mL centrifuge bottles at 500 rpm for 20 mins. Pellet was resuspended in lysis buffer (30mL 1 X PBS, 10 mM Imidazole, Protease inhibitor (cOmplete Tablets, Mini EDTA-free EASYpack Roche), 10 mM lactose, 10% glycerol). Lysate was then sonicated until culture looked translucent. Lysates were spun down in JA-20 bottles at 15,000 rpm for 40 mins at 4 °C. 250 µL Ni-NTA agarose beads were washed in lysis buffer and then mixed with lysate supernatant and incubated at 4 °C overnight.

Sample was spun down at 2000 rpm for 2 mins at 4 °C, then supernatant was removed. 5 mL wash buffer (1X PBS, 10 mM Imidazole) was added, and then spun at 2000 rpm for 1 min – this was repeated twice. 3 mL elution buffer (500 mM imidazole in PBS) was added and sample was incubated on rocker for 30 min at 4 °C before spinning at 2000rpm for 1 minute. This step was repeated, with elution being removed and saved both times. Sample was then dialysed by transferring into BioDesignDialysis Tubing (D004, Thermofisher), and floating in 3L of 1X PBS + 5% glycerol overnight on stir plate at 4 °C.

2.2.5 Ligation of DNA insert into plasmid

50 ng vector DNA was combined with 37.5 ng insert DNA and 10 µL 2X Quick Ligase reaction buffer (NEB). Nuclease free H₂O was then added up to 20 µL before adding 1 µL Quick Ligase (NEB) and gentle mixing. Mixture was incubated at room temperature for 5 minutes before being chilled on ice.

2.2.6 Ligating sgRNAs into Cas9 plasmid

Predesigned sgRNAs were ordered from IDT based off good on-target and off-target scores, (according to suggestions in Ran *et al*, 2013). Top and bottom strands of the oligos were resuspended to a final concentration of 100 μ M. Oligos were phosphorylated and annealed by mixing 1 μ L each of top and bottom strand with 1 μ L T4 ligation buffer (10x), 1 μ L T4 PNK and 6 μ L ddH₂O, and incubating in a thermocycler at 37 °C for 30 mins, 95 °C for 5 mins, then ramped down to 25 °C at 5 °C min⁻¹. Oligos were then diluted 1:200. Ligation reaction was set up by mixing 2 μ L of the oligo duplex with 100ng pSpCas9(px462) vector, 2 μ L 10X tango buffer, 1 μ L 10mM DTT, 1 μ L ATP, 1 μ L FastDigest BBPI, 0.5 μ L T4 ligase, 11.5 μ L ddH₂O. Reaction was incubated in thermocycler at 37 °C for 5 mins, 21 °C for 5 mins, for 6 cycles.

2.2.7 Restriction digest

1 μ g DNA was combined with 1 μ L restriction enzyme, 3 μ L 10X Cutsmart buffer and ddH₂O up to 30 μ L. Sample was incubated at 37 °C for at least 1 hour or overnight.

2.2.8 Gibson reaction

0.02-0.5 pmol DNA fragments were combined 1:2 or 1:3 vector to insert. DNA was then mixed with 10 μ L 2X Gibson assembly mastermix and dH₂O up to 20 μ L. Solutions were then incubated at 50 °C for 60 minutes before transformation into *E.coli* cells.

2.2.9 PCR amplification

200ng template DNA was mixed with 2.5 μ L each of forward and reverse primer (stock 10 μ M) and 10 μ L 5X Phusion HF buffer (NEB) and 1 μ L dNTPs (stock 10 mM). 0.5 μ L Phusion DNA polymerase (NEB) is added, and nuclease-free water is added up to 50 μ L. Solutions were transferred to a thermocycler, and PCR reaction carried out with the following settings:

95 °C – 1 min

95 °C – 15 sec

60 °C – 15 sec

72 °C – 2 min

35 cycles

72 °C – 5 min

4 °C - ∞

2.2.10 Colony PCR

5 colonies were picked from LB agar plates and swirled in 8 μ L dH₂O. Pipette tip was spread over section of fresh LB agar plate containing 100 μ g/mL AMP. PCR mix containing 10 μ L 2X PCRBio mix, 1 μ L U6 forward primer (10 μ M), 1 μ L reverse sgRNA (10 μ M) was made and added to the colony solution. PCR was carried out with the following settings:

95 °C – 5 min		
95 °C – 30 sec	}	30 cycles
55 °C – 30 sec		
72 °C – 1 min		
72 °C – 10 min		
4 °C - ∞		

2.2.11 DNA agarose gel electrophoresis

DNA gels were made by adding 1-3% (w/v) agarose to 1X TAE buffer, and heating with a microwave until the agarose dissolved. When solution had cooled to 50 °C, SYBRsafe is added 1:10,000, then poured into a gel tray with a well comb. DNA samples were prepared by adding 6x loading dye (NEB), and 10 μ L sample added per well along with a 1 Kb plus DNA ladder (Invitrogen). Gel box was filled with 1X TAE buffer, and gel was run at 100V until dye is ~75% through the gel.

2.2.12 Gel Extraction

Gel extraction of DNA was performed using GeneJET gel extraction kit (thermo) from 1% agarose gels separated by electrophoresis.

2.3 Western blotting

2.3.1 SDS-PAGE

SDS-PAGE gels were made using 7.5, 10, 12 or 15 % (v/v) acrylamide resolving gels with 4% stacking gels (recipe in section 2.6.1). Protein samples were mixed with 5X SDS sample buffer (6.25 mL glycerol, 12.5 mL upper Tris, 6.25 mL 1M DTT, 1.25g SDS, 62 mg bromophenol blue, dH₂O up to 25 mL) and heated at 65 °C for 5 minutes. Gels were placed in Bio-Rad mini-PROTEAN tetra cell gel apparatus (Bio-Rad) with 1X SDS running buffer (25 mM Tris, 250 mM glycine, 0.1% SDS). 10 µL All Blue Precision Plus Protein Standard (Bio-Rad) was loaded onto the gel, alongside 10 µL of each sample. Electrophoresis ran at 100V for 15 minutes, and then 150V until dye had run to the bottom of the gel.

2.3.2 Semi-dry transfer onto PVDF membrane

The gel was removed from the gel tank and prepared for transfer onto PVDF membrane using the Trans-blot SD semi-dry transfer cell system (Bio-Rad) The membrane was activated by soaking in methanol for 2 minutes. 2 pieces of filter paper were soaked in 8mL semi-dry transfer buffer (48 mM Tris, 39mM glycine, 20% MeOH, 0.0375% SDS), 30 mL MeOH, and 162 mL H₂O, and then the filter paper was placed onto the transfer plate, followed by the membrane, the gel and the 2nd filter paper. This was rolled to remove bubbles, and transfer was run at 300 mA for 60 minutes.

2.3.3 Membrane antibody probing

After transfer, the membrane was blocked in 5% milk in PBS-T (1X PBS, 0.05% Tween-20) for 1 hour. The blots were then incubated with primary antibodies in 3% milk in PBS-T overnight

at 4 °C on a platform shaker. The membrane was washed in PBS-T 3 x 10 min and then incubated with secondary antibodies in 5% milk for 1 hour at room temperature. The membrane then had 3 x 10-minute washes in PBS-T, and was imaged using a Chemi-imager (iBright FL1000, Thermofisher). Protein bands were then quantified using ImageJ, which can be used to measure the intensity of the selected bands.

2.4 Protein assays

2.4.1 Subcellular Fractionation

3x 10cm² dishes of HeLa HA-GLUT4-GFP cells were grown per condition under normal conditions with DMEM containing 10% FBS and 1% Glutamax, or with either 2 µg/mL kifunensine (Cayman chemical) or 10 µg/mL swainsonine (Cayman chemical) added for 3 days. Cells were then serum starved for 2 hours with 5 mL serum free DMEM. At 1 hour 30 minutes, cells were stimulated with 2 µM insulin for 30 minutes. Cells were placed on ice and washed 2x in 5 mL HES buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 1 mM EDTA). Cells were scraped in 1mL HES + protease inhibitor (cOmplete Tablets, Mini EDTA-free EASYpack Roche) (PI) per 3 plates (transfer buffer to next plate within same condition), and stored at -20°C.

Cells were lysed by passing them 10 times through a 25-gauge needle and twice through a 26-gauge needle, and transferred into 4.4 mL Thinwall tubes. They were then centrifuged in a TLA110 rotor at 10,000g (15,500 rpm) for 20 minutes at 4 °C. All spins were carried out with the TLA110 rotor unless stated otherwise. The supernatant was transferred into a precooled 4.4 mL thinwall tube and set aside on ice (referred to supernatant 1). The pellets were resuspended in 1 mL HES+PI and layered on top of 1 mL high sucrose HES (contains 1.12M sucrose) in a 2.2 mL thinwall tube. These were then centrifuged in a TLS-55 swing out rotor at 41,000g (25,000 rpm) for 60 minutes at 4 °C. Two layers are formed containing either high sucrose HES or HES, and the plasma membrane (PM) fraction is found between these layers. The PM fraction is removed from between the two layers using a pipette, and transferred into a clean 4.4 mL thinwall tube. The remaining pellet is resuspended in 150 µL HES+PI buffer – this is the **mitochondrial and nuclear fraction (MN)**. The PM fraction is

diluted in 4 mL HES+PI, and centrifuged at 140,000g (58,000 rpm) for 60 minutes at 4 °C. The supernatant is discarded, and the pellet is resuspended in 100 µL HES+PI – this is the **PM fraction**.

Supernatant 1 (from the first spin) was then centrifuged at 25,000g (24,500 rpm) for 20 minutes at 4 °C. The supernatant was transferred to a clean 4.4 mL thinwall tube. The pellet was resuspended in 100 µL HES+PI buffer – this is the high density microsomes (**HDM**) **fraction**.

The supernatant was centrifuged at 140,000g (58,000rpm) for 60 minutes at 4 °C. The supernatant was transferred to an Eppendorf – this is the soluble fraction. The pellet was resuspended in 100 µL HES + PI buffer – this is the low density microsomes (**LDM**) **fraction**.

2.4.2 Lactose Pulldown assay

4 confluent 10 cm² dishes of HA-GLUT4-GFP expressing HeLa cells were grown, washed twice in ice-cold 1X PBS and lysed in 1 mL 0.5% Triton-X-100/0.5% NP40 in PBS. Cells were scraped, collected in a 1.5 mL Eppendorf tube, and passed through a 23G needle. Lysate was centrifuged at 16,000g for 10 min at 4 °C, and the pellet was discarded. Concentration of lysate was checked using a BCA assay. The α-lactose-agarose beads (Sigma) or control agarose resin (Pierce) were prepared by washing 40 µL beads in 500 µL lysis buffer, centrifuging at 1000 rpm for 30 seconds and removing the supernatant. Beads were washed twice, and then lysate was incubated with the beads rotating for 1 hour at 4 °C. Beads were then washed 3x in lysis buffer to remove unbound proteins. Proteins of interest were then eluted by incubating the beads in lysis buffer + 500 mM lactose, rotating for 30 min at 4 °C. Sample was then centrifuged, and 5X sample buffer was added to sample and run on an SDS-PAGE gel.

2.4.3 BCA assay

BCA assay was performed using a BCATM Protein Assay Kit (Pierce). 2000, 1000, 500, 250, 125 and 0 µg/mL standards of BSA were made up. BCA working reagent was made by mixing reagent A and reagent B 50:1. 25 µL of each standard and the sample were pipetted into a 96-well plate, along with 200 µL working reagent. Plate was covered and incubated for 30 min at 37 °C, and then absorbance measured at 562 nm using a MULTISKAN GO plate reader (Thermo Scientific).

2.4.4 Coomassie staining

Following SDS-PAGE (section 2.3.1), gel was soaked in Coomassie stain (50% MeOH, 10% Acetic acid, 1g Coomassie in 1L) for 30 min on shaker at room temperature, and was then soaked in Coomassie de-stain (15% Acetic acid, 15% MeOH) for 2 hours, refreshing the de-stain every 30 min.

2.4.5 Silver staining

Gel and gel box were rinsed with buffer 1 (30% ethanol, 10 acetic acid in water), and gel was then soaked in buffer 1 for at least 15 minutes. Buffer 1 was removed, and buffer 2 (6mM sodium thiosulphate, 0.4 M Sodium acetate pH 6.0, 30% ethanol and 1% glutaraldehyde in dH₂O) added for 14 min. Gel was washed for 3-4 min in dH₂O x8, and then soaked in buffer 3 (6mM silver nitrate and 0.025% formaldehyde in dH₂O) for 25 minutes in dark. Gel was rinsed x2 in dH₂O, and buffer 4 (230 mM sodium carbonate and 0.04% Formaldehyde in dH₂O) was added to the gel. Gel box was gently shaken until stain began to colour, and buffer 4 refreshed 2 or 3 more times until bands appeared on the gel. Once the bands reached sufficient intensity, the reaction was stopped by adding buffer 5 (50 mM EDTA)

2.4.6 RUSH (retention using selective hooks) assay

HeLa cells were plated at 21,000 cells/well in a 24-well plate. The next day, cells were transfected with 0.5 µg HA-GLUT4-SBP-GFP or HA-GLUT1-SBP-mCherry plasmids and 1 µL XtremeGENE HP DNA transfection reagent (Sigma) in 50 µL serum-free medium (as in 2.1.9). 2 wells were treated with 2 µg/mL kifunensine and 1 well was treated with 40 µM biotin (∞). After 48 hours, 40 µM biotin was then added sequentially to wells at time points up to 60 minutes, and then placed on ice and washed 3 times in ice-cold 1X PBS. Cells were then collected in 50 µL 1x sample buffer and heated at 65 °C, in preparation for analysis by western blot.

2.4.7 Coupling of GFP-nanobody to NHS activated Sepharose beads

Following E.coli transformation and DNA miniprep of the pOpine plasmid, GFP-nanobody protein was purified from BL21 *E.coli* cells (using methods described in section 2.2). All subsequent solutions were used at 4 °C. 1 mg purified GFP-nanobody was dissolved in 0.5 mL coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3). 1 mL NHS-activated Sepharose beads was washed with 10-15 mL of cold 1 mM HCl immediately before use. Washed medium and the GFP-nanobody coupling solution were then mixed, pH adjusted to pH 6-9 and incubated overnight at 4 °C. To block non-reacted groups on the medium, GFP nanobody solution was incubated in 0.5 M Ethanolamine, 0.5 M NaCl pH 8.3 for 3 hours. GFP nanotrapp was then washed 3 times each in alternating buffers – 3 mL of 0.1M Tris-HCl buffer (pH 8-9) followed by 3 mL of 0.1 M acetate buffer (0.07M sodium acetate, 0.03M acetic acid), 0.5 M NaCl (pH 4-5). GFP nanotrapp was then stored in 20% ethanol.

2.4.8 GLUT4 purification

5x 250mL suspension flasks of HA-GLUT4-GFP HeLa cells were grown under normal growth conditions (section 2.1.1). Growth medium was removed by centrifuging at 1000rpm for 5 minutes and cells were washed 3 times in PBS. Cells were lysed in 15-20 mL lysis buffer (50 mM potassium phosphate pH 7.5, 150 mM NaCl, 15% glycerol, 0.5 mM EDTA, 1 µg/mL DNase 1, 1 mM PMSF, 0.75% Lauryl Maltose Neopentyl Glycol (LMNG)). Protein concentration was measured using a BCA assay, and amount of LMNG adjusted to 3.5 fold. Sample was then incubated for 1 hour at 4 °C, then centrifuged at 50,000 rpm at 4 °C for 45 minutes. Supernatant was filtered through a 0.45 µm filter to remove insoluble matter. 500 µL GFP-nanotrap was transferred to a 20 mL column, and pre-equilibrated with 5 mL lysis buffer. Soluble protein fraction was then loaded onto the column, and then column material washed two times with 5 mL lysis buffer containing 300 mM NaCl. Protein was then eluted with 5 mL 0.1M glycine pH 2.7 into 10 collection tubes containing 50 µL 1M Tris pH 8.5.

2.4.9 Quantification of GLUT4 using GFP standards

GFP quantification was achieved using GFP quantification kit (Abcam). GFP standards were prepared from a 10 ng/µl stock, diluted in assay buffer to create standards with 0, 80, 160, 240, 320 and 400 ng/well, with enough volume for duplicate readings. 100 µL of each standard and sample was added in a 96-well plate. Fluorescence was then measured using a Clariostar plate reader at Ex/Em = 488/507 nm.

2.5 Monosaccharide composition analysis

2.5.1 Acetone precipitation of protein sample

500 μL purified GLUT4 sample (3-5 μg) was mixed with 1 ml -20 $^{\circ}\text{C}$ acetone incubated for 60 minutes at -20 $^{\circ}\text{C}$. Sample is centrifuged for 10 minutes at 14, 000 xg, and supernatant was disposed. Residual acetone was allowed to evaporate from uncapped tube at room temperature for 30 minutes.

2.5.2 Acid hydrolysis for aldose analysis

3-5 μg precipitated GLUT4 was mixed with 75 μL ddH₂O and 100 μL TFA and mixed gently. Caps were screwed on tightly and wrapped in Teflon tape, and were then placed in a temperature controlled oven at 100 $^{\circ}\text{C}$ for 6 hours. Samples are then dried in a vacuum centrifuge overnight.

2.5.3 Re-N-Acetylation of aldoses

Hydrolysed and dried monosaccharide sample was dissolved in 200 μL saturated sodium bicarbonate solution (1M, pH 9), and cooled to 4 $^{\circ}\text{C}$. 10 μL acetic anhydride was gently added, and incubated at 4 $^{\circ}\text{C}$ for 10 minutes. This was repeated twice. Tube was warmed to room temperature over 30 minutes, and sample was then dried down in a speed-vac, re-dissolved in 50 μL dH₂O and re-dried – repeated twice.

2.5.4 Derivatization for hydrolysed aldoses

Dried hydrolysis samples were dissolved in 100 μL of 1% (w/v) sodium acetate solution and vortexed vigorously for at least 2 min. Samples were incubated at room temperature for 30 min, vortexing every 10 min to ensure the pellet is completely dissolved. 50 μL of each

hydrolysed sample were transferred to separately labelled 1.6-mL polypropylene vials. 100 μ L of anthranilic acid reagent (45mg anthranilic acid, 45mg sodium cyanoborohydride dissolved in 1.5ml of 4% (w/v) sodium acetate (trihydrate)-2% (w/v) boric acid (granular) in methanol) was added to each sample. Samples were then heated for 45 min at 80 °C. 850 μ L of solvent A (0.2% (v/v) 1 -butylamine, 0.5% (v/v) phosphoric acid, and 1.0% (v/v) tetrahydrofuran in MilliQ water) was added to each tube, then mixed and centrifuged for 5 min at maximum speed.

2.5.5 Acid hydrolysis for sialic acid analysis

50 μ L precipitated GLUT4 was mixed with 50 μ L 0.5M sodium bisulphate and mixed gently. Samples are then heated to 80 °C for 20 minutes, then removed from heat and allowed to cool to room temperature.

2.5.6 Derivatization for hydrolysed sialic acid

100 μ L o-Phenylenediamine (OPD) solution (20 mg/mL OPD in 0.25M sodium bisulphate) is added to 100 μ L sialic acid working solution (1 mM sialic acid in 0.05% acetic acid diluted 1:100 with 0.25M sodium bisulphate) and hydrolysed sample then mixed gently. Samples are heated at 80 °C for 40 minutes, before removing and allowing to cool to room temperature. 800 μ L of solvent A (0.2% (v/v) 1 -butylamine, 0.5% (v/v) phosphoric acid, and 1.0% (v/v) tetrahydrofuran in MilliQ water) was added to each tube, then mixed and centrifuged for 5 min at maximum speed.

2.5.7 Methods for HPLC

Table 2-1 - Reverse-phase HPLC gradient conditions for aldose analysis

Time	Flow (ml/min)	% Solvent B (rest is solvent A)
0	0.6	5
7	0.6	5
16	0.6	6.5
28	0.6	8
28.5	0.6	100
33	0.6	100
33.5	0.6	5
42	0.6	5

Table 2-2 - Reverse-phase HPLC gradient conditions for sialic acid analysis

Time	Flow (ml/min)	% Solvent B (rest is solvent A)
0	0.6	10
15	0.6	10
15	0.6	95
25	0.6	95
25	0.6	10
35	0.6	10

2.6 Materials

2.6.1 Solutions:

10X PBS – 27 mM KCl, 1.37 M NaCl, 100 mM Na₂HPO₄, 20 mM KH₂PO₄, pH adjusted to 7.4 with NaOH

PBST – 1X PBS with 0.1 % Tween-20

Blocking solution for western blotting - 5% (w/v) dried milk in PBST

Blocking solution for fluorescence staining – 2% (w/v) BSA (Tocris) in 1X PBS

Coomassie stain – 50% (v/v) Methanol, 10% (v/v) Acetic acid, 0.1% (w/v) coomassie brilliant blue R-250

Coomassie de-stain – 15% (v/v) Acetic acid, 15% (v/v) Methanol

5x Sample buffer - 6.25 mL glycerol, 12.5 mL upper Tris, 6.25 mL 1M DTT, 1.25g SDS, 62 mg bromophenol blue, dH₂O up to 25 mL

Resolving buffer (Lower Tris, pH 8.8) – 1.5 M Tris, 0.4% (w/v) SDS

Stacking buffer (Upper Tris, pH 6.8) – 0.5 M Tris, 0.4% (w/v) SDS

Table 2-3 - SDS-PAGE gel recipe (for 2 gels)

Percentage	7.5%	10%	12%	15%	4% (Stacking)
H ₂ O (mL)	6	5.1	4.2	3	2.7
Resolving/Stacking buffer (mL)	3	3	3	3	1.125
30 % Acrylamide (mL)	3	3.9	4.8	6	0.6
10% APS (μL)	100	100	100	100	40
TEMED (μL)	20	20	20	20	10

SDS-PAGE running buffer - 25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS

Semi-dry transfer buffer - 48 mM Tris, 39 mM glycine, 20% (v/v) MeOH, 0.0375% (w/v) SDS

LB Medium – 2.5% (w/v) LB in dH₂O

LB agar – 2.5% (w/v) LB, 1.5% Agar in dH₂O, 1:1000 antibiotic (added after autoclaving)

TAE – 40 mM Tris-Acetate pH 8, 1 mM EDTA

HES buffer – 250 mM sucrose, 20 mM HEPES (pH 7.4), 1 mM EDTA

High sucrose HES buffer – 1.12M Sucrose in HES buffer

2.6.2 Mammalian cell lines

HA-GLUT4-GFP HeLa adherent cells

HA-GLUT4-GFP HeLa suspension cells

GFP CHO cells

HeLa cells

3T3-L1 HA-GLUT4-GFP

HA-GLUT4-GFP HeLa Δ Gal-3

2.6.3 Competent *E.coli* strain

BL21 (NEB)

DH5 α (NEB)

2.6.4 Antibodies

Table 2-4 Primary antibodies

Antigen	Working dilution	Description	Source
HA.11	FACS: 1:1000 IB: 1:1000	Mouse, monoclonal	Biolegend (901514) Stock = 1-3 mg/mL
Galectin-1	FACS: 1:1000 IB: 1:1000	Mouse, monoclonal	Proteintech (60223-1-Ig) Stock = 2.3 mg/mL
Galectin-3	FACS: 1:1000 IB: 1:1000	Mouse, Monoclonal	Santa Cruz Biotechnology (sc-32790) Stock = 200 µg/mL
Beta-Tubulin	IB: 1:1000	Mouse, monoclonal	Proteintech (66240-1-Ig) Stock = 2 mg/mL
GFP	IB: 1:1000	Rabbit, monoclonal	Eurogentec (gift from Paul Pryor)
mCherry	IB: 1:1000	Mouse, monoclonal	Sigma (SAB2702291) Stock = 1 mg/mL
LAMP1 (H4A3)	IB: 1:1000	Mouse, monoclonal	Developmental Studies Hybridoma Bank (university of Iowa)
IRAP	IB: 1:500	Rabbit, polyclonal	Cell Signalling (3808)
EEA1	IB: 1:10,000	Mouse, monoclonal	BD transduction laboratories (610456) Stock = 250 µg/mL
TGN46	IB: 1:1000	Rabbit, polyclonal	Millipore (ABT95)

GM130	IB: 1:1000	Mouse, monoclonal	BD Transduction laboratories (610822) Stock: 250 µg/mL
Calreticulin	IB: 1:1000	Rabbit, polyclonal	Pierce (PA3-900)
Streptavidin	IB: 1:1000	Rabbit, whole antiserum, polyclonal	Sigma (S6390)

Table 2-5 - Secondary antibodies

Antigen	Working dilution	Description	Source
Mouse HRP	IB: 1:2000	Horse, IgG	Cell signalling (7076S)
Mouse AlexaFluor 647	FACS: 1:500	Goat, IgG	Invitrogen (A-21235) Stock = 2 mg/mL
Rabbit HRP	1:2000	Goat, IgG	Cell signalling (7074S)

2.6.5 DNA vectors and inserts

Table 2-6 - DNA plasmids

Plasmid	Description	Source
Gal1-6xHis (NdeI-XhoI)	Ampicillin resistant <i>E.coli</i> expression vector, expressing Galectin-1 with a His-tag	Dr Christian Wunder (Blouin et al., 2016)
05162 pOPINE2 GFP-nanobody	Ampicillin resistant <i>E.coli</i> expression vector, expressing GFP-nanobody with His-tag	Constructed by Dr Oleksiy Kovtun (gift from Paul Pryor)
HA-GLUT4-SBP-GFP RUSH plasmid	Ampicillin resistant <i>E.coli</i> expression vector	Brodsky Lab (Camus et al., 2020)
HA-GLUT1-SBP-mCherry RUSH plasmid	Ampicillin resistant <i>E.coli</i> expression vector	Brodsky Lab (Camus et al., 2020)
pcDNA3	Ampicillin resistant <i>E.coli</i> expression vector	Gift from Richard Ingram
HA-GLUT4 pBabe-puro	Retroviral	Constructed by Dr Rebecca McCann (Lamb et al., 2010)
HA-GLUT4(7K/R) pBabe-puro	Retroviral	Constructed by Dr Rebecca McCann (Lamb et al., 2010)

Table 2-7 - Oligonucleotides (purchased from IDT)

Oligo	Sequence
Galectin-1 sgRNA forward	5' – AAA CGG AGA GTG CCT TCG AGT GCG C – 3'
Galectin-1 sgRNA reverse	5' – CAC CGC GCA CTC GAA GGC ACT CTC C – 3'
Galectin-3 sgRNA forward	5' – CAC CGC TGC ACC TGG AGT CTA CCC A – 3'
Galectin-3 sgRNA reverse	5' – AAA CTG GGT AGA CTC CAG GTG CAG C – 3'
SBP knock-in forward sgRNA 1	5' – CACCg AAAGGAGAAGAAGCTTTTCAC – 3'
SBP knock-in reverse sgRNA 1	5' – AAAC GTGAAAAGTTCTTCTCCTTTc – 3'
SBP knock-in forward sgRNA 2	5' – CACCg GCACAGAAGCTTGAGTATTTA – 3'
SBP knock-in reverse sgRNA 2	5' – AAAC TAAATACTCAAGTTCTGTGCc – 3'
SBP repair template	5' - TTCCACCGGACACCCTCTCTTTTAGAGCAGGAGGTGAA <u>ACCTAGCACAGAAGCTTGAGTATTTA</u> AGGGCCAGATGAG AACGACATGGACGAGAAGACCACTGGTTGGCGAGGTG GACACGTTGTTGAAGGACTGGCTGGGGAAGTTGAACA ACTTCGTGCACGACTGGAGCATCACCCACAAGGTCAAC GTGAACCAATGAGTAAAGGAGAAGAGCTCTTTACTGG AGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAA T – 3'
HA-GLUT4 or HA-GLUT4(7K/R) Gibson PCR primer forward	5' - CTT GGT ACC GAG CTC GGA TCA TGC CGT CGG GCT TCC AAC A - 3'
HA-GLUT4 or HA-GLUT4(7K/R) Gibson PCR primer reverse	5' - TGA TGG ATA TCT GCA GAA TTC TCA GTC GTT CTC ATC TGG CC - 3'

HA-GLUT4/HA-GLUT4(7K/R)_pcDNA3 primer forward	5' – GTA GGC GTG TAC GGT GGG AGG T– 3'
HA-GLUT4/HA-GLUT4(7K/R)_pcDNA3 primer reverse	5' – GGC GAA CGT GGC GAG AAA GGA A – 3'

3 Analysing the functional effects of altering

GLUT4's *N*-glycan structure

GLUT4's amino acid motifs that regulate various stages of the transporter's trafficking itinerary, along with the molecular machinery with which they interact, have been well-characterised since GLUT4 was first identified (Bryant et al., 2002; Lalioti et al., 2001). In contrast, less is known about the effect of post-translational modifications, such as *N*-glycosylation, on the regulation of GLUT4's insulin-responsive trafficking (Sadler et al., 2013). Although studies on the role of GLUT4's single *N*-glycan indicate a function in the trafficking of GLUT4, there is little consensus about the precise stage at which it is required (Haga et al., 2012; Haga et al., 2011; Hirayama et al., 2016; Ing et al., 1996; Zaarour et al., 2012). This is in part due to the varied techniques and model systems used in the different studies. This chapter presents data from a systematic analysis, using well-characterised tools and reagents, to study the function of GLUT4's *N*-glycan in regulating its trafficking itinerary.

GLUT4 expression is limited to muscle and fat cells, but numerous studies have demonstrated that GLUT4 is trafficked in a regulated manner in most, if not all, cell types when ectopically expressed, indicating that the molecular machinery required for insulin-regulated GLUT4 traffic is ubiquitously expressed (Bryant and Gould, 2020). This important observation opens up the possibility of using cell types that are more experimentally tractable than terminally differentiated fat and muscle cells to study GLUT4 traffic. The majority of experiments undertaken in this project utilise a HeLa cell line which stably expresses HA-GLUT4-GFP. This cell line has previously been used to study GLUT4 trafficking (Camus et al., 2020; Haga et al., 2011; Kioumourtzoglou et al., 2015). It has been shown to

share very similar trafficking kinetics to 3T3-L1 adipocytes, and has a comparable insulin response via the same pathway (Morris et al., 2020). HeLa cells are also much easier to use, as they do not have to undergo differentiation, and are also robust, fast-growing and amenable to genetic manipulation.

One of the methods used in previous studies to investigate GLUT4's *N*-glycan is the mutation of GLUT4's glycosylation site (N57Q) to prevent addition of the *N*-glycan (Haga et al., 2011; Zaarour et al., 2012). While this allows the specific abolishing of GLUT4's *N*-glycan rather than causing global glycosylation defects, *N*-glycans often play a role in protein folding and quality control mechanisms in the ER, so mutating the glycosylation site may interfere with these.

The aim of this chapter is to use two glycosylation inhibitors to analyse the effect of altering the *N*-glycan structure. These inhibitors avoid affecting protein folding and quality control as they act after the protein has left the ER. The first of these inhibitors is kifunensine which has been used in previous studies and has been shown to have no effect on GLUT4 stability (Haga et al., 2011; Hirayama et al., 2016). It inhibits the glycosylation enzyme mannosidase I (MAN1), which trims the mannose residues before further processing can occur (**Figure 3-1**). By inhibiting MAN1, all Golgi processing is blocked, and only Man₉GlcNAc₂ remains attached to the protein.

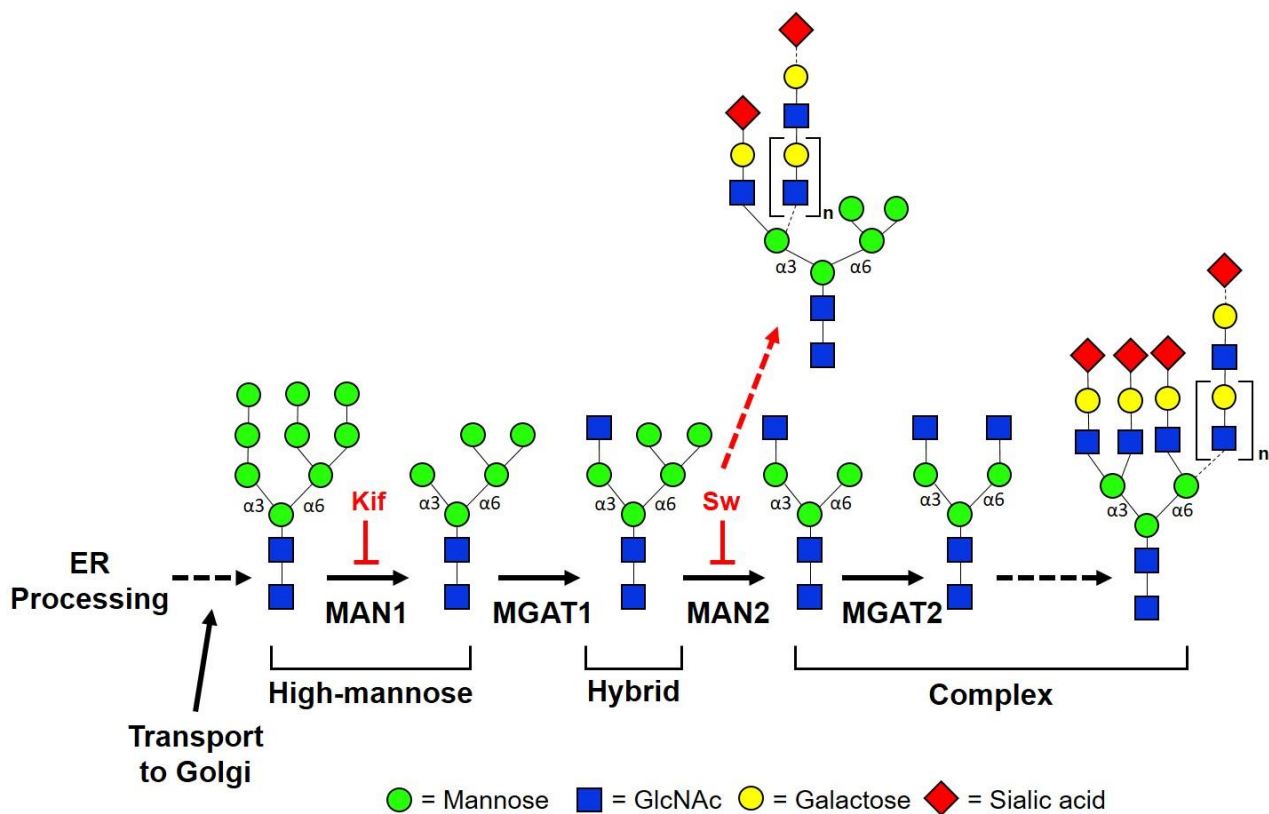


Figure 3-1 - Glycan processing in the Golgi

Following the initial transfer of an N-glycan to the protein and subsequent trimming in the ER, the protein is transported to the Golgi carrying a Man₉GlcNAc₂ N-glycan. Trimming of 4 mannose units by mannosidase I (MAN1) is followed by the addition of a GlcNAc unit onto the α 3 branch by MGAT1. Mannosidase II (MAN2) then trims a further 2 mannose units from the α 6 branch, allowing MGAT2 to add a GlcNAc unit, forming a complex glycan which can undergo further processing to add additional monosaccharide units. Kifunensine (Kif) inhibits the action of Mannosidase I (MAN1) and Swainsonine (Sw) inhibits Mannosidase II (MAN2) in the trimming of mannose units on one branch – thereby preventing the formation of the complex glycan, and forming a structure whereby polylactosamine is added to the α 3 branch.

Work presented in this chapter also used swainsonine, an inhibitor of mannosidase II (MAN2). MAN2 acts later on in glycan processing to remove two mannose residues and enable the formation of a complex N-glycan (**Figure 3-1**). Consequently, swainsonine treatment inhibits the transition of an N-glycan from a hybrid to a complex form. This is important as this conserves more of the original structure than kifunensine, allowing a more nuanced approach to altering the N-glycan.

For example, a common feature of complex N-glycans is the presence of an extended polylactosamine chain (composed of repeating galactose and GlcNAc subunits). Under normal conditions, this is preferentially added to the $\alpha 6$ branch (Pierce and Arango, 1986; Yamashita et al., 1984). However in the presence of swainsonine, the processing of the $\alpha 6$ branch is inhibited, and polylactosamine is added onto the $\alpha 3$ branch instead (Elbein et al., 1981) (**Figure 3-1**). This means that swainsonine treatment may not significantly change the size of the N-glycan even if the structure is altered, because the same features may still be present.

3.1 Results

3.1.1 GLUT4 has a single large *N*-glycan

It is well-documented that GLUT4 has a single large *N*-glycan (Haga et al., 2011; Ing et al., 1996; Zaarour et al., 2012). Treatment of HA-GLUT4-GFP HeLa cell lysates with PNGaseF, which acts on denatured protein to cleave off *N*-glycans, results in a significant reduction of the molecular weight of GLUT4 (**Figure 3-2a&b**). This size shift was quantified to be around 25-35 kDa. To control for any effects of overnight incubation on protein degradation, samples without PNGaseF treatment were collected and stored immediately at -20°C, or incubated overnight. The protein size was the same with and without overnight incubation, indicating little to no protein degradation (**Figure 3-2**). This shows that the molecular weight decrease was solely due to the loss of an *N*-glycan that is around 25-35 kDa. This is considered large, as the maximum size of a fully processed *N*-glycan without any polymers is around 4 kDa.

This suggests that there may be either a polylactosamine chain or a polysialic acid chain present on GLUT4's *N*-glycan. Polysialic acid is an unusual modification which is primarily been found in bacteria or in the mammalian nervous and immune systems (Colley et al., 2014; Villanueva-Cabello et al., 2021). Polylactosamine is therefore more likely to be present, although structural analysis is needed to confirm this.

This also establishes that the HA and GFP tags do not interfere with the glycosylation of GLUT4 in HeLa cells, in which HA-GLUT4-GFP has previously been demonstrated to traffic in an insulin-regulated manner indistinguishable from endogenously expressed GLUT4 in fat and muscle cells ((Haga et al., 2011; Morris et al., 2020; Muretta et al., 2008).

In addition to the PNGase F treatment on cell lysates, HA-GLUT4-GFP HeLa cells were also treated with kifunensine or swainsonine prior to cell lysis. Kifunensine treatment results in a large decrease in the molecular weight of GLUT4 similar to that of PNGase F, indicating that the bulk of the N-glycan has been removed (**Figure 3-2a**). In contrast, swainsonine treatment does not dramatically alter the molecular weight of GLUT4, indicating that the bulk of the N-glycan is still there (**Figure 3-2b**). If swainsonine has been effective in these cells in inhibiting MAN2, polylactosamine may have been added on the $\alpha 3$ branch as the preferred $\alpha 6$ branch won't be accessible for polylactosamine addition (**Figure 3-2**) (Elbein et al., 1981). This means that, while the size is the same, the specific structure may be altered.

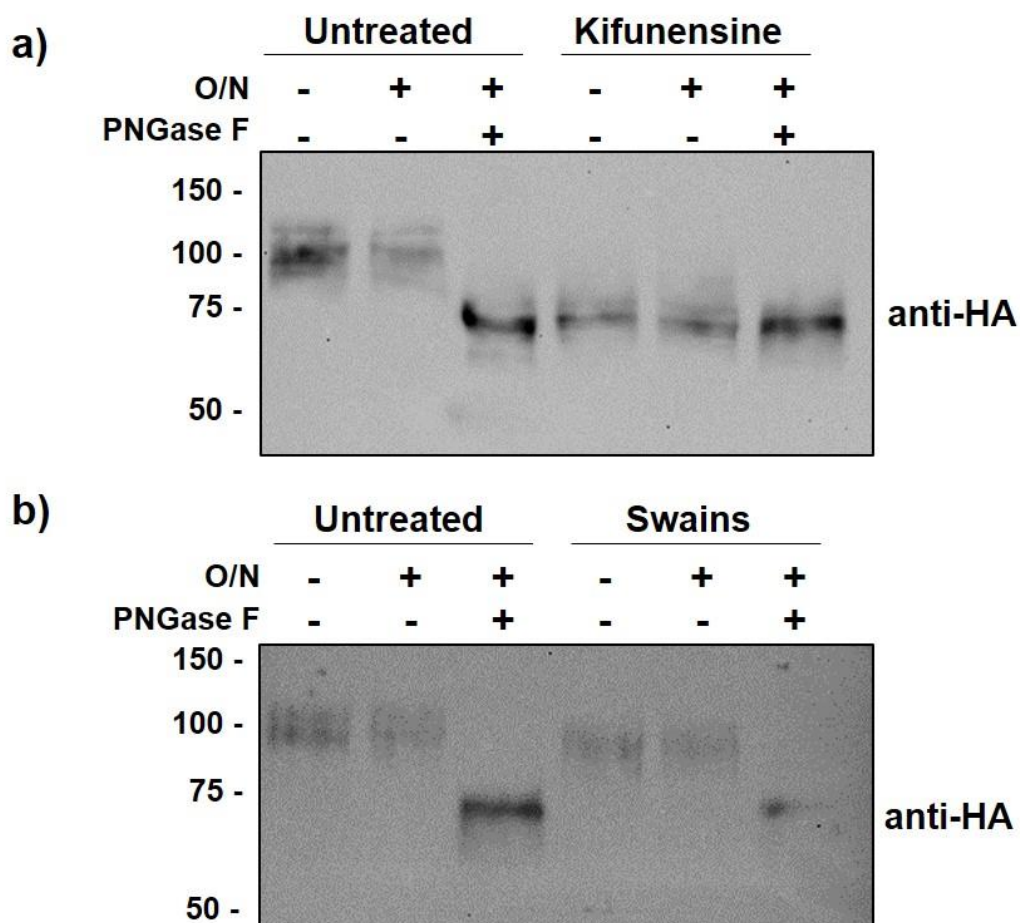


Figure 3-2 - GLUT4 has a single large N-glycan

a&b) Cell lysates were collected following kifunensine (**a**) or swainsonine (**b**) incubation, then either treated at 37°C with PNGaseF overnight, incubated overnight at 37°C with no PNGaseF treatment, or had no PNGaseF treatment and no 37°C incubation. Samples were then subjected to western blot analysis (using an anti-HA.11 antibody) following separation on a 10% SDS-PAGE gel.

3.1.2 Altering GLUT4's *N*-glycan causes an increase in surface GLUT4 levels

To investigate the effect of kifunensine and swainsonine treatment on surface GLUT4, an established protocol for quantifying surface GLUT4 as a proportion of total GLUT4 levels was used (Camus et al., 2020). This utilises the tags in the HA-GLUT4-GFP construct (**Figure 3-3a**). In short, a cell-impermeable anti-HA antibody labels surface GLUT4, which is then bound by a fluorescence labelled secondary antibody. This fluorescent signal representing surface GLUT4 is measured alongside the GFP signal, which represents total GLUT4 (**Figure 3-3b**). During flow cytometry, HA and GFP signals are gated to find the ratio of median HA signal:median GFP signal, representing surface GLUT4 as a proportion of total GLUT4 (**Figure 3-3c**).

To confirm that cells were not permeabilised in the fixation process, the assay was performed with DAPI (a non-permeable stain), with and without permeabilisation using saponin (**Figure 3-3d**). Cells without saponin had no significant difference in DAPI signal compared to the “no DAPI” control, whereas the cells that had been permeabilised had a 2.5x increase in DAPI signal. This indicates that the fixation process did not result in permeabilisation of the cells, so the HA antibody will only stain the GLUT4 present at the cell surface.

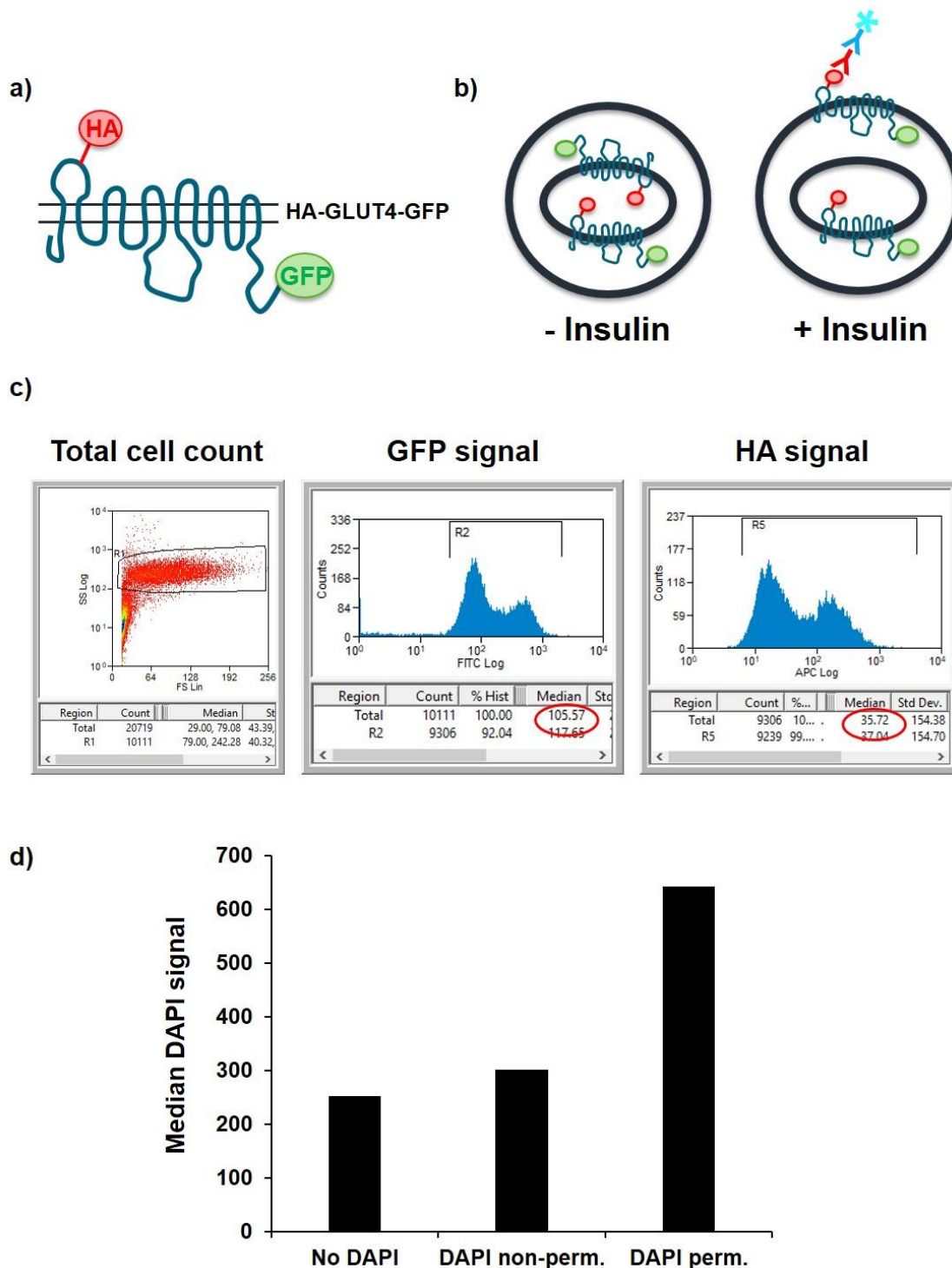


Figure 3-3 – Surface GLUT4 can be selectively labelled using an anti-HA antibody

a) Diagram of GLUT4, the 7-transmembrane spanning glucose transporter with a HA tag on the first extracellular loop and a GFP tag on the intracellular c-terminus **b)** An anti-HA.11 antibody binds to HA at the extracellular face, which is then bound by a fluorescent secondary antibody, thereby selectively visualising surface GLUT4 **c)** Once cells are fixed and fluorescently stained, they are collected for flow cytometry. Healthy cells can be selected through gating (as in left side graph), and then further gating selects the population of cells with GFP and HA signal. The median for each of these is recorded to find the HA:GFP ratio representation the proportion of GLUT4 at the cell surface. **d)** HA-GLUT4-GFP HeLa cells were fixed in 2% PFA, then one well was permeabilised using 0.1% saponin. 2 wells were stained with DAPI. DAPI signal was then measured by flow cytometry.

In order to observe the effect of altering the *N*-glycan structure on surface GLUT4 levels, HA-GLUT4-GFP HeLa cells were treated with kifunensine or swainsonine for 48 or 72 hours respectively, followed by treatment with or without insulin for 30 minutes. Surface GLUT4 levels were then measured by flow cytometry (**Figure 3-4**). As expected, insulin treatment caused a significant increase in surface GLUT4 compared to the untreated cells. Both kifunensine and swainsonine caused a significant increase in surface GLUT4 under basal conditions, and a reduction of the insulin response. This indicates that removing the bulk of GLUT4's *N*-glycan results in increased surface GLUT4, a finding consistent with previous studies (Haga et al., 2011; Hirayama et al., 2016).

While swainsonine has no effect of the mobility of GLUT4 when separated by SDS-PAGE, the effect on surface GLUT4 levels suggests that it has altered the structure of the *N*-glycan. This shows that rather than an *N*-glycan size requirement, a specific *N*-glycan structure is important to function in the intracellular sequestration of GLUT4 in the absence of insulin. This is a key observation that underscores the importance of determining the *N*-glycan structure attached to GLUT4 (which is explored in chapter 4).

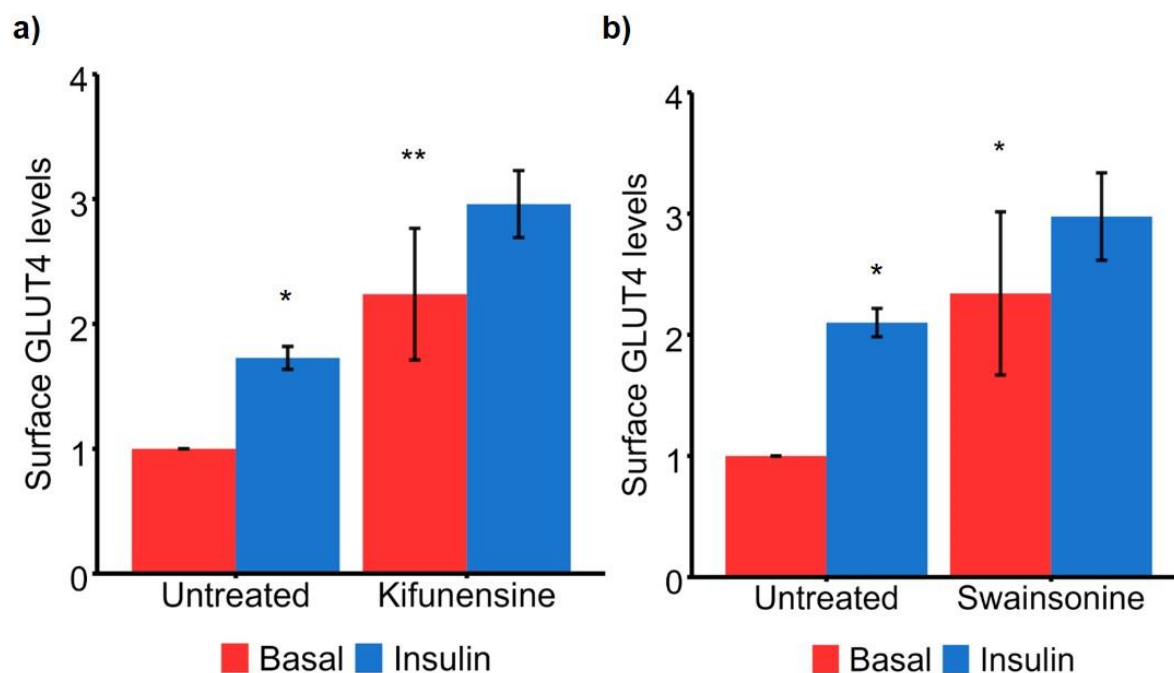


Figure 3-4 - Kifunensine and swainsonine increase surface GLUT4 levels

HA-GLUT4-GFP HeLa cells were incubated with either **a)** kifunensine (2 μ g/mL, incubated overnight) or **b)** swainsonine (10 μ g/mL, incubated for 72 hours). Cells were serum starved with serum free DMEM only for 2 hours, with insulin (1 μ g/mL) added to cells at 90 minutes. Cells were fixed in 2% PFA, then surface stained with α -HA antibody (1:1000) and α -mouse Alexa 647 (1:500). HA:GFP ratio was measured by flow cytometry to show the proportion of GLUT4 present at the surface. Data is normalised to basal untreated cells, n=5. Significance values compared against basal untreated, ** = $p < 0.01$, * = $p < 0.05$

3.1.3 Subcellular distribution of GLUT4 is affected by alterations to *N*-glycan

In order to more fully characterise the effect of kifunensine and swainsonine on GLUT4 localisation across the cell, subcellular fractionation of HA-GLUT4-GFP HeLa cells was performed. This is a well-established method for observing the movement of GLUT4 across different fractions (representing different subcellular compartments) (Black et al., 2022; Koester et al., 2022; Sadler et al., 2016). These fractions include: low density microsomes (LDM – containing recycling endosomes, Golgi elements, and IRVs), high density microsomes (HDM – containing endosomes, Golgi and ER), plasma membrane (PM), and a mitochondrial and nuclear fraction (MN).

The schematic for this fractionation experiment is shown in **Figure 3-5**, and was performed on lysates taken from cells treated with kifunensine, swainsonine or untreated cells, with or without insulin stimulation. Protein amounts were measured by BCA assay and adjusted to give an equal amount of protein across treatments within each fraction – this was verified by coomassie stain (**Figure 3-6**). This allows treatments to be compared against each other within each fraction, but not between fractions. The relative amount of GLUT4 and the indicated marker proteins in each fraction was visualised using western blot (**Figure 3-6**).

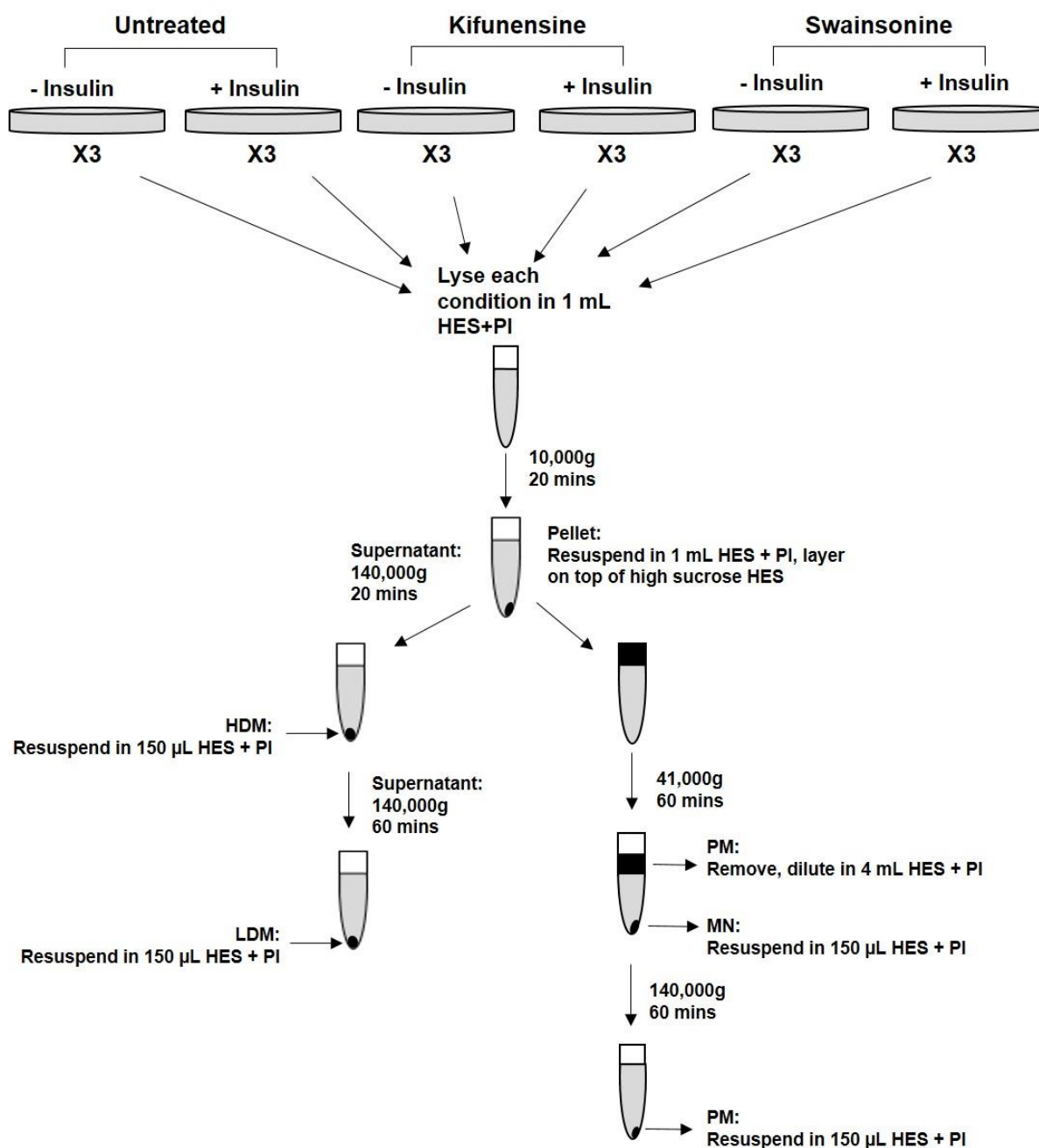


Figure 3-5 – Subcellular fractionation schematic to separate a lysate into its subcellular compartments

Cell lysates from Untreated, kifunensine and swainsonine treated cells (all + or – insulin) were collected and centrifuged at 10,000 $\times$ g for 20 minutes. The supernatant was then resuspended and centrifuged at 140,000 $\times$ g for separate into the HDM (pellet) and LDM (supernatant). The LDM supernatant was spun at 140,000 $\times$ g again to separate into the soluble fraction (supernatant) and the LDM (pellet). The pellet from the original spin was resuspended and layered on top of high sucrose HES, then spun at 41,000 $\times$ g to separate the PM (between the two layers) and the MN (pellet) fractions. Adapted from Sadler et al, 2016.

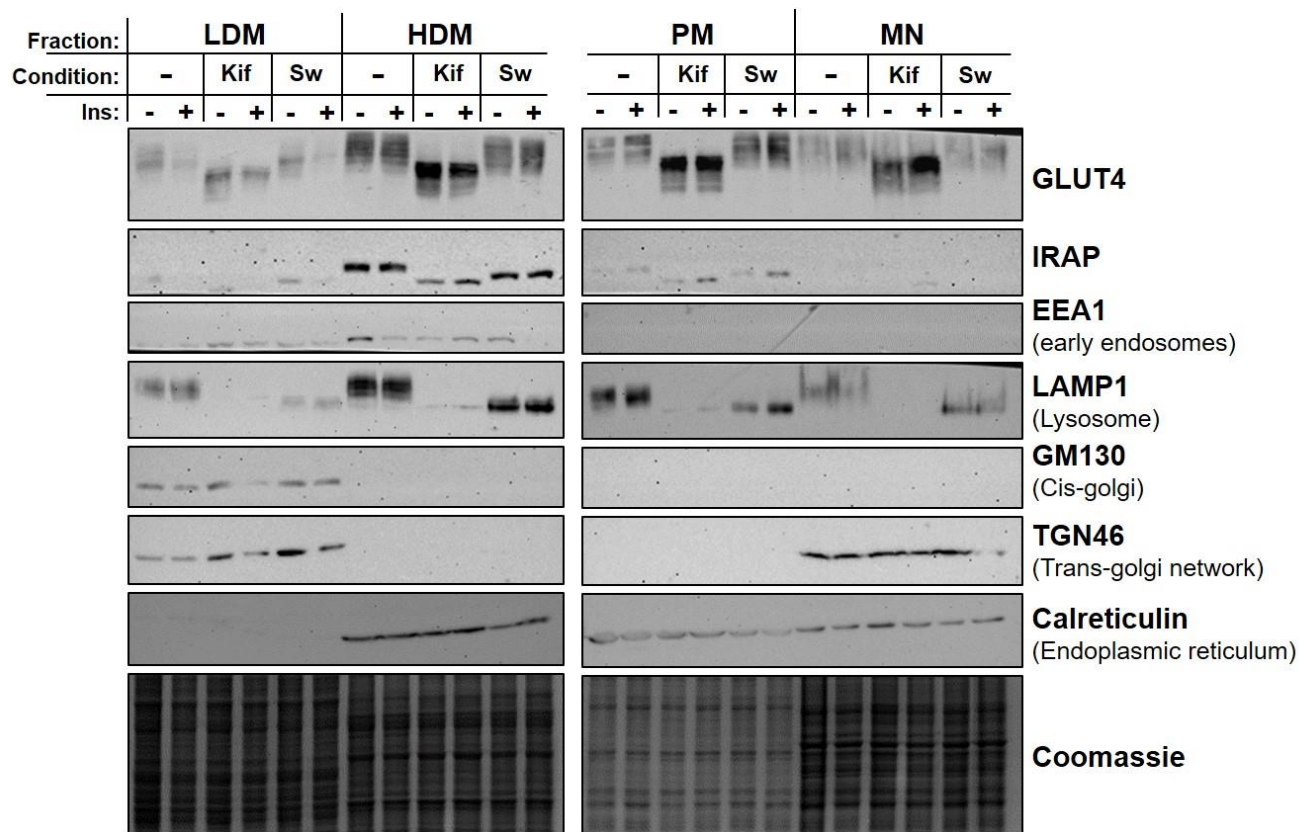


Figure 3-6 – Subcellular fractionation of HA-GLUT4-GFP HeLa cells shows changed localisation of GLUT4 with altered N-glycan

Cells were treated with kifunensine (2 μ g/mL), swainsonine (10 μ g/mL) or left untreated for 72 hours. Cells were then serum starved for 2 hours, with some cells being stimulated with insulin (1 μ m) at 90 minutes. Lysates were then collected, and fractionation was performed. Samples were then separated on a 12% SDS-PAGE gel and subjected to western blot analysis with anti-GLUT4, anti-IRAP anti-EEA1, anti-LAMP1, anti-GM130, anti-TGN46 and anti-calreticulin. Lysates were also stained with coomassie following separation by SDS-PAGE to compare protein levels across each fraction. LDM = low density microsomes, HDM = high density microsomes, PM = plasma membrane, MN = mitochondrial and nuclear fraction. Blot is representative image of 5 biological fractionation repeats.

Quantification of immuno-reactive GLUT4 bands across 5 biological repeats showed that GLUT4 from untreated cells acts as previously reported in regards to insulin stimulation (Figure 3-7) (Black et al., 2022; Koester et al., 2022). Specifically, insulin treatment results in an increase in GLUT4 band intensity in the plasma membrane, and a concomitant decrease in the LDM fraction, which contains the IRVs (Hashiramoto and James, 2000; Piper et al., 1991; Simpson et al., 1983). This reflects the movement of GLUT4 from its intracellular store to the cell surface with insulin stimulation (Bryant et al., 2002), providing confidence that the fractionation has worked as previously reported. In accordance with data shown in Figure 3-4, kifunensine and swainsonine treatment cause an increase in the amount of GLUT4 in the plasma membrane fraction compared with untreated, and a loss of insulin response (Figure 3-6 and Figure 3-7). While the swainsonine effect is not as large as kifunensine, this increase in surface GLUT4 is consistent with the effect observed using flow cytometry (**Figure 3-4**). This provides further evidence that the *N*-glycan plays a role in GLUT4's sequestration away from the cell surface in the absence of insulin. However, despite the loss of insulin response at the plasma membrane with kifunensine and swainsonine treatment, a loss of GLUT4 from the LDM following insulin treatment is still observed, raising questions as to where the GLUT4 goes (**Figure 3-7**). Due to time restraints, this observation was not investigated further.

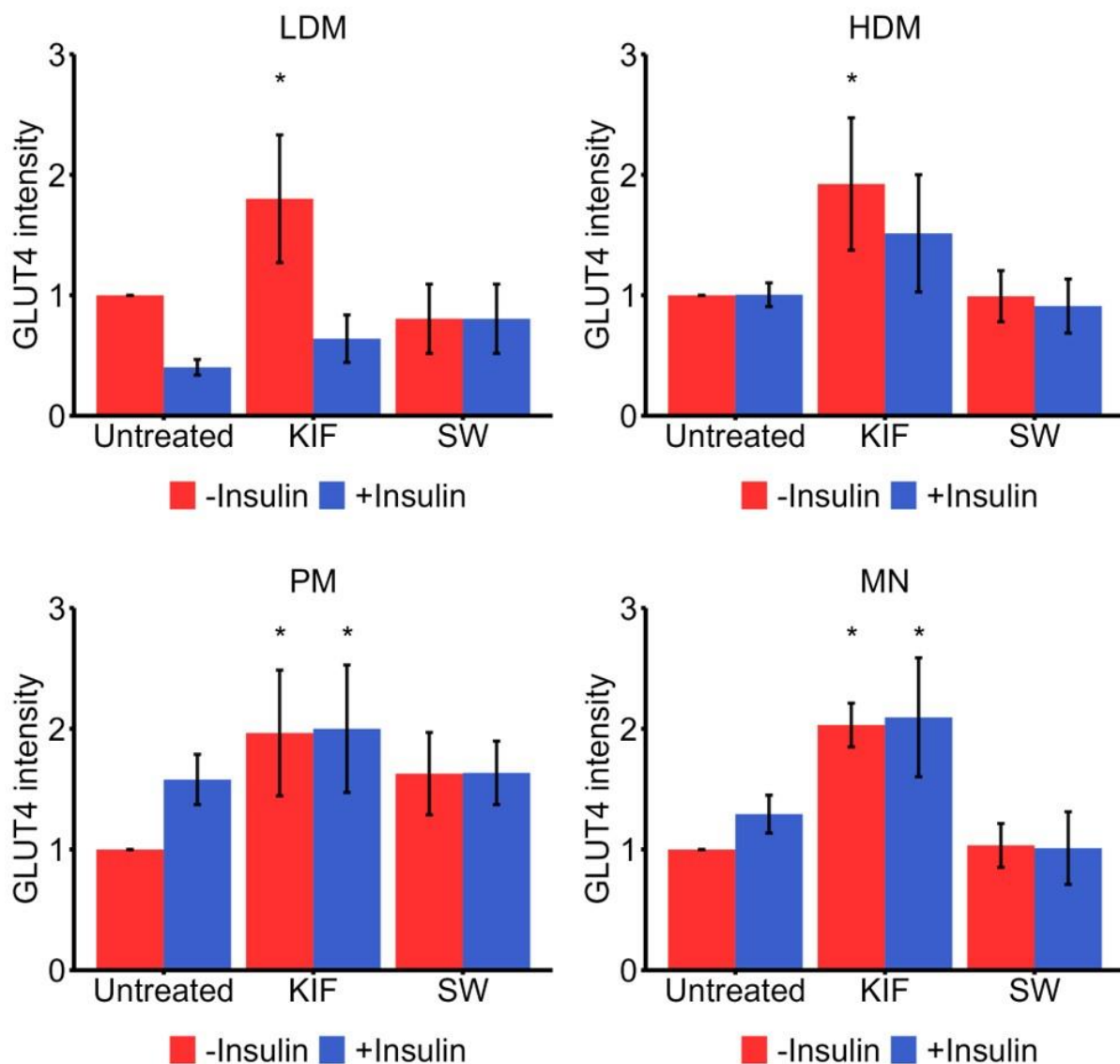


Figure 3-7: Quantification of GLUT4 bands within each subcellular fraction

GLUT4 band intensities were measured using ImageJ across 5 separate experiments, * = $p < 0.05$. LDM = low density microsomes, HDM = high density microsomes, PM = plasma membrane, MN = mitochondrial and nuclear fraction.

It is important to note that neither kifunensine nor swainsonine have a global effect of insulin-responsive GLUT4 trafficking. As mentioned in section 1.2.2, IRAP (insulin responsive aminopeptidase) is an insulin responsive protein with the same trafficking itinerary as GLUT4 (Jordens et al., 2010; Ross et al., 1996). It is also *N*-glycosylated, as shown by the decrease in molecular weight following both kifunensine and swainsonine treatment (Parker et al., 2016) (**Figure 3-6**). In contrast to GLUT4, there is no significant increase of IRAP at the PM with kifunensine or swainsonine treatment and no loss of insulin response (**Figure 3-6**).

Another glycoprotein that was blotted against was LAMP1, which was used as a lysosomal marker (although it is found across many of the endosomal compartments including the PM) (Cheng et al., 2018). Although this has not been reported previously, there is an increase of LAMP1 in the PM with insulin stimulation (**Figure 3-6** and **appendix I**). This is also seen in MSC-derived adipocytes, indicating that it demonstrates insulin-responsive trafficking in physiological conditions (**Appendix I**). In contrast to GLUT4, kifunensine treatment resulted in a large decrease in LAMP1 intensity across each fraction, suggesting that the protein is degraded in the absence of its glycans (**Figure 3-6** and **appendix I**). This is consistent with previous studies which show that glycosylation plays a protective role in LAMP1 (Kundra and Kornfeld, 1999).

Together, the effects seen for both IRAP and LAMP1 suggest that GLUT4's *N*-glycan has a specific function not seen across all insulin-responsive glycoproteins, and that the effects observed in GLUT4 aren't caused by the global *N*-glycosylation changes.

In contrast to insulin treatment which increases the amount of GLUT4 in the PM fraction but decreases the amount in the LDM, kifunensine treatment of cells appears to increase the

amount of GLUT4 in all fractions (Figure 3-7). This is not consistent with a simple change of localisation, but instead implies an overall increase in total levels of GLUT4 within the cell. Kifunensine and swainsonine treatment of whole cell lysates showed that kifunensine caused a significant increase in GLUT4 amount (**Figure 3-8**). In contrast, swainsonine treatment resulted in no significant change in GLUT4 levels (**Figure 3-8**). While swainsonine treatment retains the size of the *N*-glycan, kifunensine treatment causes a significant decrease in size, reducing it to the Man₉GlcNAc₂ structure (Figure 3-1). This could indicate that the *N*-glycan has a second role in regulating GLUT4 function that is size-dependent rather structure-dependent.

A hypothesis for this second role is that *N*-glycan size is a marker for determining GLUT4 age. It is known that GLUT4 continuously cycles through the endosomal system, including the TGN (Martin et al., 2000b; Ralston and Ploug, 1996; Shewan et al., 2003; Slot et al., 1997; Slot et al., 1991a; Slot et al., 1991b). It is possible that GLUT4 undergoes further *N*-glycan processing each time it travels through the TGN, until it reaches a certain size above which it is targeted for degradation.

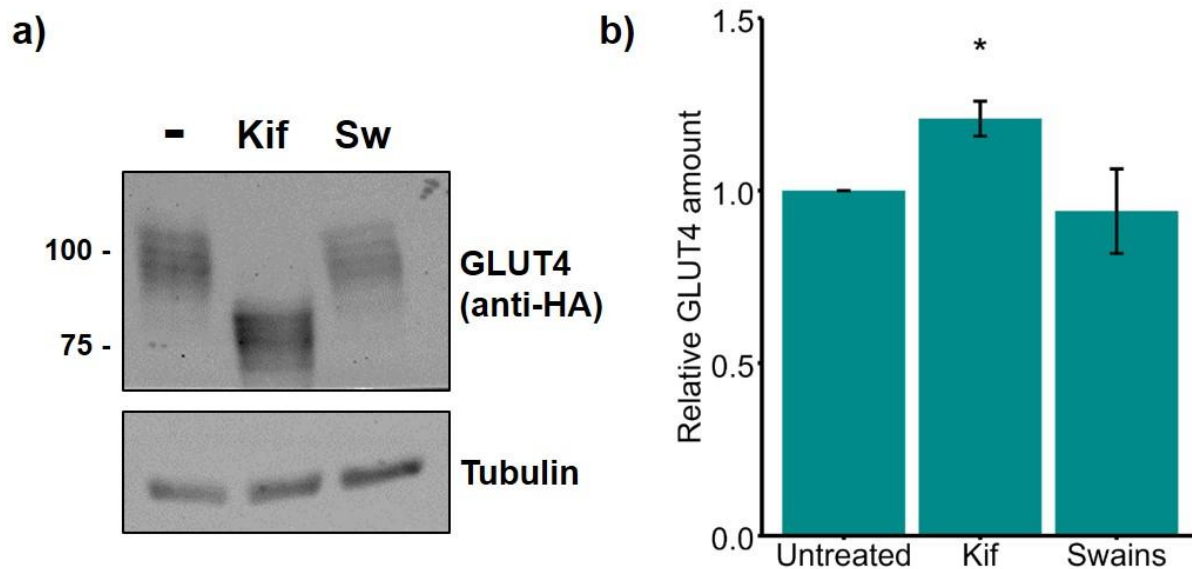


Figure 3-8 – Kifunensine treatment results in increased amount of GLUT4

a) HeLa HA-GLUT4-GFP cells were treated with 2 $\mu\text{g}/\text{mL}$ Kifunensine or 10 $\mu\text{g}/\text{mL}$ Swainsonine for 72 hours. Samples were subjected to western blot analysis (using anti-HA.11 and anti-tubulin antibodies) following separation on a 7.5% SDS-PAGE **b)** GLUT4 band intensities were measured across 6 experiments using ImageJ, * = $p < 0.05$

3.1.4 Transfection of cells with HA-GLUT4 & HA-GLUT4(7K/R)

To test the hypothesis that *N*-glycan length is an indicator of GLUT4 age, the band pattern of HA-GLUT4 was compared with a ubiquitin-resistant version of HA-GLUT4. A previous member of the lab (Rebecca McCann) generated a HA-GLUT4(7K/R) mutant, in which all of the ubiquitination sites are mutated (Lamb et al., 2010). Ubiquitination is required for both GLUT4 degradation and its insulin response (Lamb et al., 2010). The loss of insulin response was proposed to be due to a loss of sorting into IRVs, with GLUT4(7K/R) instead being held in the TGN (Lamb et al., 2010).

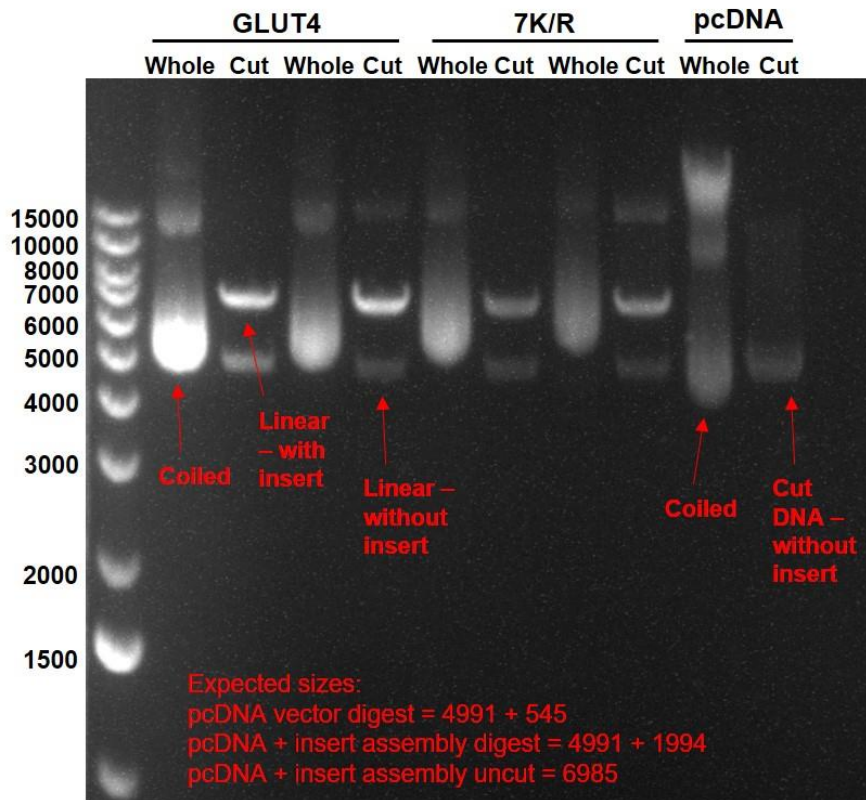
The previously generated HA-GLUT4 and HA-GLUT4(7K/R) plasmids were pBabe-puro retroviral plasmids designed for viral transduction, and so were not suitable for transient transfection. Gibson assembly was used to insert the HA-GLUT4 and HA-GLUT4(7K/R) DNA inserts into a pcDNA3 plasmid which can be transfected into HeLa cells. A diagnostic restriction digest was performed using restriction sites either side of the DNA insert, and samples were run on a DNA agarose gel (**Figure 3-9a**). The bands observed on the gel suggested that some DNA was only digested by one enzyme, leaving a linearised DNA band the size of the vector + insert (6985 bp), and a DNA band the size of the vector without the insert (4991 bp). The size shift between cut and uncut DNA was equal to the size of the insert (1994 bp), showing that insertion was successful.

HA-GLUT4 and HA-GLUT4(7K/R) plasmids were then transiently transfected into HeLa cells. After 24 hours, samples were collected and visualised by western blot analysis (**Figure 3-9b**). A large decrease in molecular weight was observed for HA-GLUT4(7K/R) compared to HA-GLUT4, similar to the size shift observed when GLUT4's *N*-glycan is removed. This size

shift is not due to the loss of ubiquitin, as this did not affect the molecular weight of GLUT4 in previous studies (Lamb et al., 2010).

Loss of ubiquitination causes the retention of GLUT4 in the TGN, so GLUT4's normal recycling loop is prevented and it only passes through the TGN once (Lamb et al., 2010). If the decrease in GLUT4 molecular weight is due to a loss of *N*-glycan, this would suggest that GLUT4 undergoes sequential *N*-glycan processing each time it passes through the TGN and that GLUT4's blocking the recycling loop prevents GLUT4's *N*-glycan reaching its full size. This would support a hypothesis that the size of GLUT4's *N*-glycan acts as an age indicator. PNGase F treatment of the HA-GLUT4(7K/R) sample would confirm whether the decrease in size is linked to the loss of *N*-glycosylation. However, time constraints prevented this experiment being completed.

a)



b)

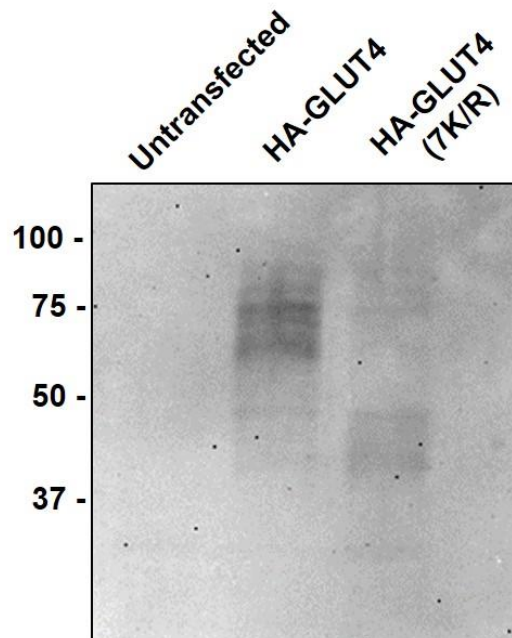


Figure 3-9- Gibson assembly of HA-GLUT4 and HA-GLUT4(7K/R) into pcDNA3

a) Following Gibson assembly of HA-GLUT4 and HA-GLUT4(7K/R) inserts into pcDNA3, a diagnostic restriction digest was performed to confirm that insertion was successful. Following restriction digest, samples were loaded onto a 1% agarose gel and subjected to DNA electrophoresis. Red arrows indicate sizes of cut and uncut plasmids **b)** Following transient transfection of HA-GLUT4 and HA-GLUT4(7K/R) into HeLa cells, cell lysates were subjected to western blot analysis with anti-HA.11 (1:1000) following separation on 10% SDS-PAGE gel

3.1.5 RUSH assay with HA-GLUT4-SBP-GFP and HA-GLUT1-SBP-mCherry

To further investigate this hypothesis, a RUSH (Retention Using Selective Hooks) assay was used to track the glycosylation of GLUT4 over time as it moves through the endosomal system. This assay works by transiently expressing a plasmid which encodes HA-GLUT4-SBP-GFP (or HA-GLUT1-SBP-mCherry) and a streptavidin-tagged ER resident protein (Str-II). Streptavidin binds to SBP (streptavidin binding protein), but is outcompeted by biotin. When excess biotin is added, GLUT4 is released from the ER as one homogeneous group, and it is possible to track its progression through the endosomal system (as represented in **Figure 3-10**). These plasmids were gifted by the Brodsky lab, who have previously published data demonstrating the successful retention and release of the GLUT4 and GLUT1 using microscopy (Camus et al., 2020).

Following transfection of the RUSH plasmids and a biotin time course, lysates were collected and subjected to western blot analysis in order to observe the molecular weight shift over time (**Figure 3-10b&c**). In **Figure 3-10b**, a significant difference is seen in the molecular weight of GLUT1 between no release (–) and continuous biotin (∞), demonstrating that the protein has been glycosylated and the assay works as expected. This glycosylated form of GLUT1 starts to appear at 30 minutes, but there is still a large proportion of the non-glycosylated form at 60 minutes, indicating that a longer time frame is needed to observe the complete glycosylation of GLUT1.

While the different glycosylation states of GLUT1 are clear, this was not the case for GLUT4 (**Figure 3-10c**). Initial transfection attempts were unsuccessful, and transfection time was extended to 72 hours which improved protein expression (data not shown). However, even

for cells treated with biotin for the whole 72 hours (∞), there was only small proportion of GLUT4 at the higher molecular weight expected (~120 kDa), suggesting that it may take longer than 72 hours for GLUT4 to undergo *N*-glycan processing. There were also bands at high molecular weights across all samples that were not present in untransfected cells (**appendix II**), which could indicate issues with the RUSH plasmid itself.

Generating a RUSH HA-GLUT4-SBP-GFP & Str-II stable cell line avoids the need for transient transfection and allow GLUT4 to be observed over longer time periods. A strategy was developed to stably transfect Str-II into HA-GLUT4-GFP HeLa cells, and add an SBP tag to HA-GLUT4-GFP using CRISPR-Cas9n knock in. Although Str-II was successfully stably expressed in the cells, time constraints prevented this experiment being completed (see **Appendix III**).

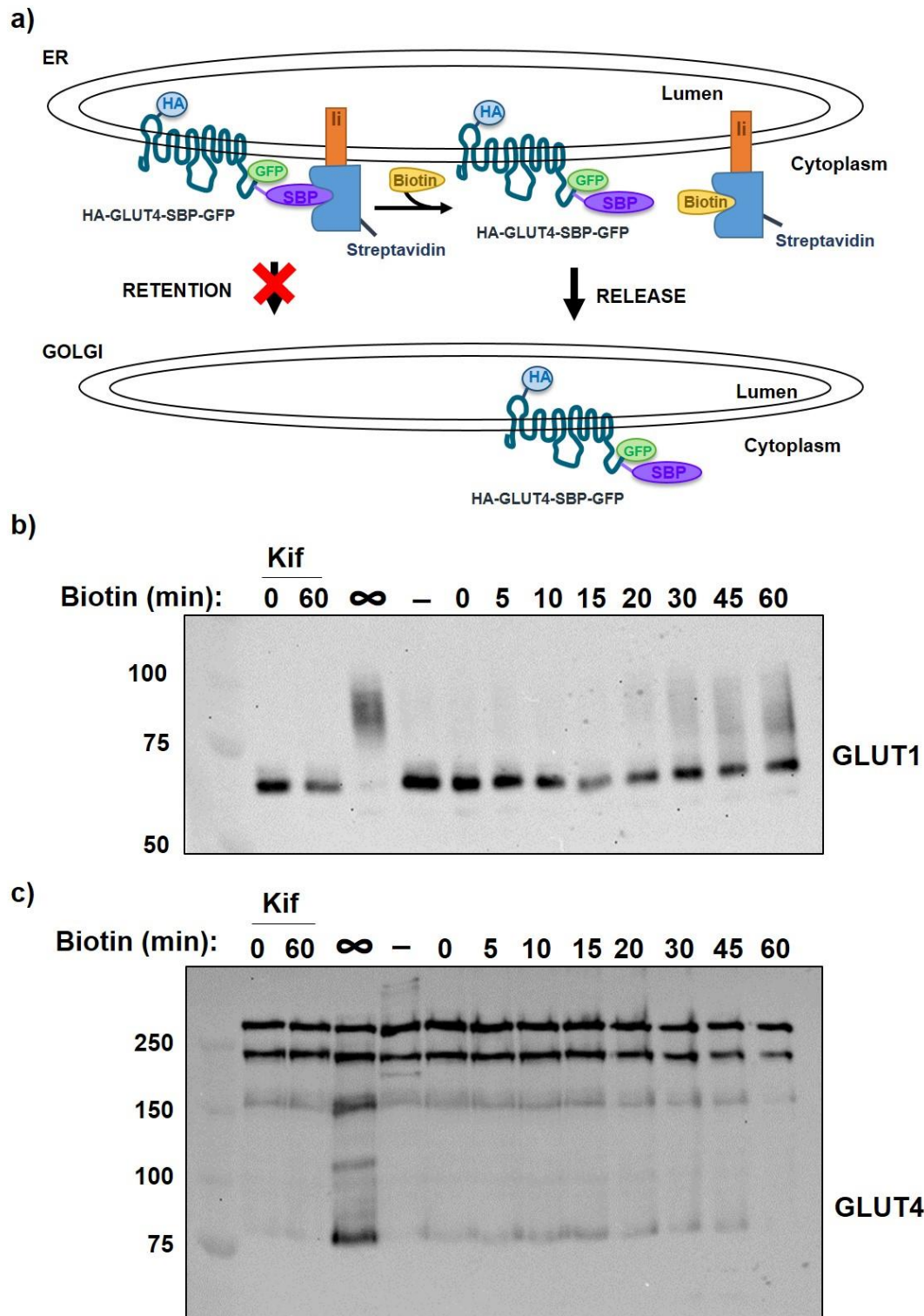


Figure 3-10 – RUSH assay shows changes in glycosylation over time

a) Diagram representing the mechanism of GLUT4 retention and release in the RUSH system **b&c)** HeLa cells were transiently transfected with the RUSH plasmids (HA-GLUT1-SBP-mCherry or HA-GLUT4-SBP-GFP). 2 wells were treated with 2 μ g/mL kifunensine (kif), and 1 well was treated with 40 μ M biotin (∞). After 48 hours (GLUT1) or 72 hours (GLUT4), 40 μ M biotin was added to the cells for 0, 5, 10, 15, 20, 30, 45 and 60 minutes. (–) represents no addition of biotin. Cells were then placed on ice and lysed in 1X sample buffer. Samples were run on 7.5% SDS-PAGE gel and subjected to western blot analysis with anti-mCherry or anti-HA.

3.2 Discussion

This chapter reports findings using an established model system (HA-GLUT4-GFP HeLa cells) and two glycosylation inhibitors to study the role of GLUT4's *N*-glycan. This revealed two potential roles of the *N*-glycan – one in the regulation of surface expression of GLUT4 in the absence of insulin, and one in the degradation of GLUT4.

Previous studies investigating the role of GLUT4's *N*-glycan have struggled to come to a consensus on the role of the *N*-glycan (Haga et al., 2011; Hirayama et al., 2016; Ing et al., 1996; Zaarour et al., 2012). One of the aspects that may contributed to this confusion is the use of a GLUT4(N57Q) mutant to remove the glycosylation site (Haga et al., 2011; Ing et al., 1996; Zaarour et al., 2012). While this allows for the specific removal of GLUT4's *N*-glycan rather than a global inhibition of *N*-glycan processing, it can cause complications. Multiple studies observed that levels of GLUT4(N57Q) are significantly reduced compared with the wild-type protein (Haga et al., 2011; Ing et al., 1996; Zaarour et al., 2012), which is consistent with the known role that *N*-glycans have in protein stability. Using the N57Q mutant may therefore obscure any later effects on GLUT4 trafficking due to low expression of GLUT4. Glycosylation enzyme inhibitors act after the protein has left the ER and don't affect protein stability (Haga et al., 2011), so are an improved method of studying protein glycosylation.

Analysis of surface GLUT4 levels using both flow cytometry data and subcellular fractionation revealed that altering the structure of the *N*-glycan results in an increase of GLUT4 at the plasma membrane. Data from previous studies is consistent with this finding. For example, using a cell surface biotinylation assay, Haga *et al* observe an increase in

surface GLUT4 in basal conditions for both GLUT4(N57Q) and kifunensine treated GLUT4 compared with WT GLUT4. Zaarour *et al* also observe a larger amount of surface GLUT4(N57Q) compared to WT GLUT4 in basal conditions using a fluorescence based assay. However, both studies focus on the insulin response, so this isn't followed up in either study.

Together, these data indicate that one role for GLUT4's *N*-glycan is in the intracellular sequestration of GLUT4 in basal conditions, either through decreased exocytosis or increased endocytosis. This is seen after swainsonine treatment despite the molecular weight remaining the same, indicating that a structural change has occurred which is crucial to its function. This highlights the need for the structural analysis of the *N*-glycan, which is explored in the following chapter.

In addition to revealing a role for GLUT4's *N*-glycan at the cell surface, subcellular fractionation and whole cell lysate analysis also revealed that kifunensine, but not swainsonine, caused an increase in the steady state levels of GLUT4 (**Figure 3-7 & Figure 3-8**). This indicates there may be a second function of the *N*-glycan that is dependent more on its size than the specific structure. This was not observed in previous studies – again this is likely due to the focus on the protein levels of WT GLUT4 vs GLUT4(N57Q), in which the effects on protein stability can obscure other effects.

GLUT4 has been shown to traffic through the TGN as part of its recycling pathway in adipocytes and muscle cells, which is an unusual pathway for membrane proteins (although not unique) (Martin *et al.*, 2000b; Ralston and Ploug, 1996; Shewan *et al.*, 2003; Slot *et al.*, 1997; Slot *et al.*, 1991a; Slot *et al.*, 1991b). The purpose of this trafficking has remained unclear beyond it being part of a loop which excludes GLUT4 from the cell surface in basal

conditions. A hypothesis to explain GLUT4's increased abundance following kifunensine treatment data is that as GLUT4 passes through the TGN, the *N*-glycan becomes further processed by the glycosylation enzymes present in the TGN. This processing transfers additional monosaccharide molecules to the chain, increasing *N*-glycan size each time it completes a recycling loop. The size of the *N*-glycan could act as an "age indicator", where once the *N*-glycan exceeds a certain size, it becomes targeted for degradation.

Consequently, removing the bulk of GLUT4's *N*-glycan with kifunensine no longer targets the protein for degradation, explaining the increased abundance. This would also account for why this effect is not observed in swainsonine, as although the structure is altered, swainsonine does not affect the size of the *N*-glycan.

GLUT4 is an essential protein in maintaining whole body glucose homeostasis, and its dysregulation causes insulin resistance and type 2 diabetes (Stenbit et al., 1997). It would be worthwhile to the health of the body to ensure that the pool of GLUT4 is functional, in order to rapidly and effectively respond to high glucose levels in the body. This model would allow GLUT4 to be released from the recycling loop once it reaches a certain age to limit the amount of older and potentially damaged GLUT4 present in the cell.

Data which support this hypothesis have been observed in IRAP, a protein that follows a very similar trafficking pathway to GLUT4. When IRAP was treated with neuraminidase, an enzyme which removes terminal sialic acid units, re-sialylation is observed over time (Shewan et al., 2003). This shows that it is capable of undergoing glycan processing following retrograde trafficking back to the TGN and supports the idea that GLUT4's *N*-glycan may undergo further processing as it goes through each recycling loop.

Two strategies were pursued to test this hypothesis. The first of these made use of a plasmid expressing ubiquitin-resistant GLUT4, which was originally made by Rebecca McCann by mutating 7 lysine residues to arginine. This was used in a study which demonstrated roles for ubiquitination both in targeting GLUT4 for degradation and in its insulin response – specifically the sorting of GLUT4 into insulin responsive vesicles (Lamb et al., 2010). Transiently transecting HA-GLUT4 and HA-GLUT4(7K/R) into HeLa cells revealed a significant decrease in the molecular weight of GLUT4 in the absence of ubiquitin. Together with the knowledge of GLUT4's continuous transport through the TGN, this data can be explained by the fact that GLUT4's recycling loop through the TGN is impeded in the 7K/R mutant, which prevents any additional glycosylation processing. This supports a hypothesis for the sequential *N*-glycan processing of GLUT4 which eventually leads to its degradation. Further evidence for this would include the PNGase F treatment of HA-GLUT4(7K/R) to confirm whether the decrease in molecular weight is indeed due to the loss of *N*-glycan.

The second strategy which was pursued to test the hypothesis was to use the “Retention Using Selective Hooks” (RUSH) assay to track one pool of GLUT4 over time. However, this strategy could not be completed, due to issues with transfection and time constraints for creating a stable RUSH cell line. Completing this experiment would be a key priority for future work, as it would allow for the observation of GLUT4 *N*-glycosylation over time, to see whether this happens in a sequential manner.

In summary, GLUT4's *N*-glycan contributes to two separate processes; the first is the intracellular sequestration of GLUT4, in which *N*-glycan structure plays a key role, and the second is an age-limiting degradation mechanism dependent on its size.

4 Structural analysis of GLUT4's *N*-glycan

Although little information is known about the structure of GLUT4's *N*-glycan, it was demonstrated in the previous chapter to be fundamental to its role in the intracellular sequestration of GLUT4. Furthermore, a previous GLUT4 study demonstrated that sialylated *N*-glycan has different internalisation kinetics to non-sialylated *N*-glycan, further highlighting the importance of the structure in its function (Haga et al., 2012).

It has been shown both in previous studies and the previous chapter that the *N*-glycan is large, as seen by the molecular weight shift of HA-GLUT4-GFP when the *N*-glycan is removed (**Figure 3-2**) (Haga et al., 2011; Zaarour et al., 2012). The *N*-glycan can also be assumed to be in the complex form because swainsonine, which inhibits the transition of a hybrid *N*-glycan to a complex form, has a functional effect on GLUT4 (**Figure 3-4**). However, little is known about the composition of the *N*-glycan. The aim of this chapter is to begin the structural analysis of GLUT4's *N*-glycan through monosaccharide composition analysis.

As in the previous chapter, the HA-GLUT4-GFP HeLa cell line was used. This model system demonstrates insulin-stimulated trafficking of GLUT4 to the cell surface via the same mechanism as adipocytes (Morris et al., 2020). Furthermore, data presented in the previous chapter show that the HA and GFP tags do not interfere with GLUT4's *N*-glycosylation (3.1.1). This demonstrates that HA-GLUT4-GFP HeLa cells are a good model system for studying GLUT4 trafficking, and are much easier to grow in large quantities, which is necessary for the purification of GLUT4 and subsequent structural analysis. Finally, consistency across experiments allows the functional experiments to be directly linked with any structural findings, avoiding the confusion of using different cell lines.

4.1 Results

4.1.1 Generation of a GFP nanotrap for GLUT4 purification

Monosaccharide composition analysis requires purified GLUT4 in order to release the monosaccharides from its *N*-glycan. A method of purifying HA-GLUT4-GFP from HeLa cells was therefore developed, using previous studies to optimise solubilisation of GLUT4 and GFP-nanotrap based protein purification (Kraft et al., 2015; Rothbauer et al., 2008). A GFP-nanotrap is made through coupling GFP nanobodies to Sepharose beads, which can be used in the purification of GFP-tagged proteins from mammalian cells (Rothbauer et al., 2008). Nanobodies are small single domain antibody fragments made up of the V_HH variable region of heavy chain homodimers – a form of antibody that is produced naturally in camelids (Fridy et al., 2014). These antibody fragments can be easily expressed in bacteria for protein production, and bind to their antigen with picomolar affinity (Fridy et al., 2014).

The GFP nanobody was purified from BL21 *E.coli* cells following expression of the pOpine GFP nanobody plasmid (Kubala et al., 2010) (**Figure 4-1a**). This achieved a high level of purity, and the concentration of the GFP nanobody was determined to be 2.5 mg/mL by comparing coomassie stained protein bands with BSA standards (**Figure 4-1b**). The nanobody was then coupled to N-hydroxysuccinimide-Sepharose (NHS) beads to create the nanotrap. Through incubating GFP expressing CHO cell lysates with the GFP nanotrap beads, they were shown to be highly effective at binding soluble GFP, indicating that the coupling reaction was successful (**Figure 4-1c**).

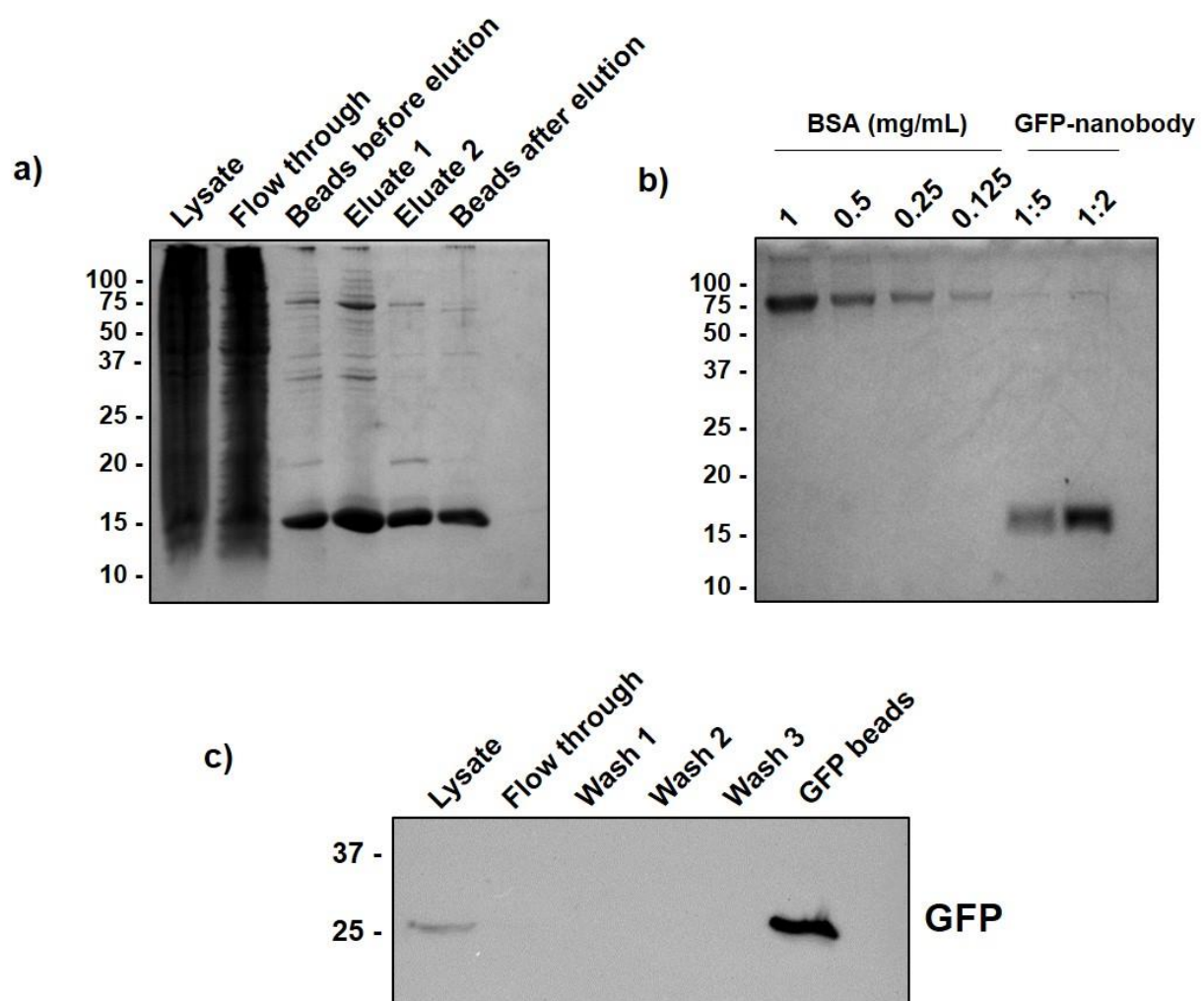


Figure 4-1 - GFP-nanobody purification

a) GFP nanobody was purified from BL21 E.coli cells expressing a pOpine plasmid, by pulldown with Ni-agarose beads. Samples from GFP-nanobody purification were run on a SDS-PAGE gel and coomassie stained. **b)** GFP-nanobody concentration was determined by running on a 15% SDS-PAGE gel alongside BSA standards and visualisation with Coomassie Blue staining. Band intensity was analysed using ImageJ, which determined GFP-nanobody concentration is 2.5 mg/mL **c)** Pulldown using CHO cells expressing GFP. Western blot analysis was performed with anti-GFP (1:1000) on samples separated by 15% SDS-PAGE

4.1.2 Optimisation of GLUT4 solubilisation

Using starting conditions from a study which optimised the purification of FLAG-tagged GLUT4 from mammalian HEK293 cells, a method for HA-GLUT4-GFP purification was developed (Kraft et al., 2015). This study identified Lauryl Maltose Neopentyl Glycol (LMNG) as the most effective at solubilising GLUT4 from a range of detergents, so this was used in this purification.

Measuring protein concentration for each purification was crucial to calculating the correct amount of detergent. BCA assays were carried out with PBS only, lysis buffer or lysis buffer + LMNG to assess its suitability for determining protein concentration. The standard curves generated were almost identical, showing that neither the lysis buffer nor the detergent affected protein concentration measurements (**Figure 4-2**)

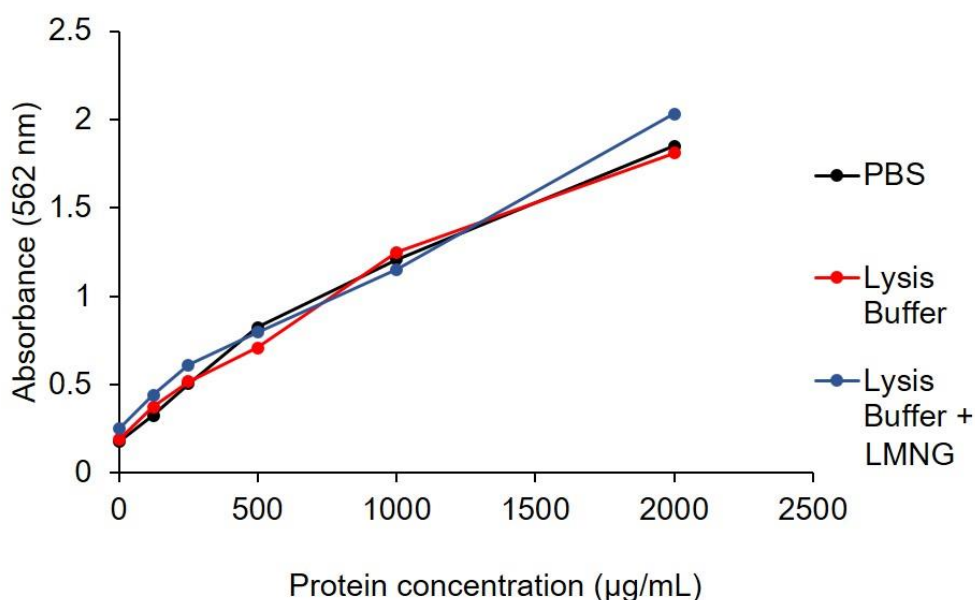


Figure 4-2 - BCA assay optimisation

BCA assay was carried out using Pierce BCA assay kit. 3 sets of standards were made up in i) BSA + PBS ii) BSA + lysis buffer (50mM potassium phosphate, 150 mM NaCl, 15% glycerol, 0.5 mM EDTA, 1 µg/mL DNase I, 1 M PMSF) iii) BSA + lysis buffer + 1% LMNG. BSA standards (0, 125, 250, 500, 1000, 2000 µg/mL) were mixed with working reagent and incubated for 30 mins at 37 °C, then absorbance was measured on a plate reader.

A general method was devised based on two previous studies, as described in **Figure 4-3a** (Kraft et al., 2015; Rothbauer et al., 2008). One key step of this method is the solubilisation of GLUT4, so the conditions for this step were optimised. Different concentrations of NaCl were tested in the solubilisation of HA-GLUT4-GFP HeLa lysates, along with different concentrations of detergent (LMNG) relative to protein concentration. Protein concentration was measured by BCA assay, and normalised to between 2.5-3.5 mg/mL. Concentration of LMNG was then measured at different ratios (2.5x, 3.5x and 5x). Centrifugation of the lysate separated GLUT4 into solubilised (supernatant) and non-solubilised (pellet) fractions. Solubilisation was then assessed by comparing the amount of GLUT4 in the supernatant to the pellet by western blot analysis (**Figure 4-3b&c**). Quantification of this data shows that 150 mM NaCl and 3.5-fold LMNG compared to protein concentration generates the greatest yield (**Figure 4-3c**).

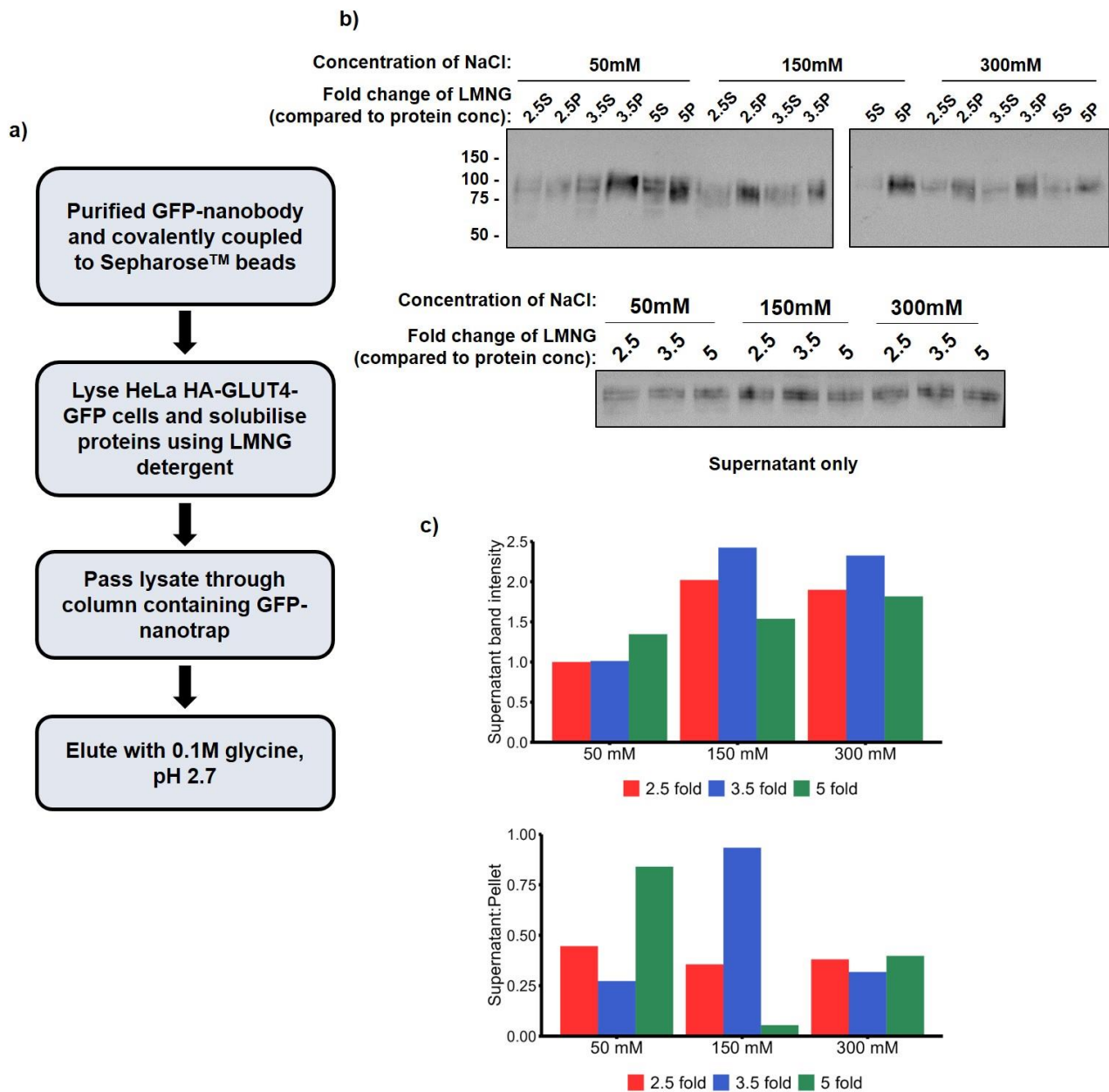


Figure 4-3 - GLUT4 purification optimisation

a) GLUT4 purification method flow **b)** HA-GLUT4-GFP HeLa cells were lysed with different concentrations of NaCl and fold change of LMNG compared to protein concentration (measured by BCSA), and a western blot was run with both the supernatants and pellets, and supernatants only, using α -HA antibody (1:1000). S=supernatant P=pellet **c)** Quantification of supernatant: pellet ratio

Purification of GLUT4 was performed using the conditions identified in **Figure 4-3**. This showed that GLUT4 purification was successful, as demonstrated by western blot analysis (**Figure 4-4a**). However, GLUT4 could not be detected by silver stain (**Figure 4-4b**) indicating a low yield, and preventing the ability to assess purity. Consequently, further optimisation of GLUT4 purification was required.

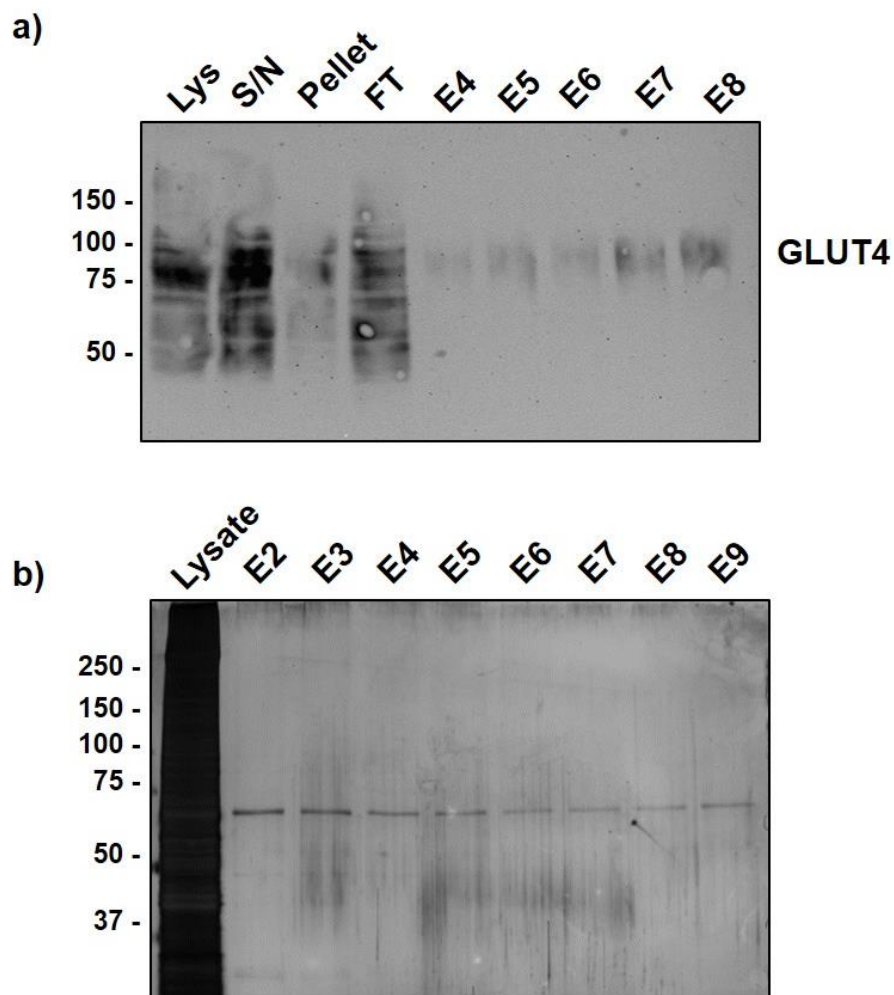


Figure 4-4 - GLUT4 purification with low yield

GLUT4 was purified from HA-GLUT4-GFP HeLa cells using a GFP nanotrap as described in figure 4.3a. Samples were run on 10% SDS-PAGE gel: Lys (lysate), S/N (supernatant), pellet, FT (flow through) and E2-9 (elutions). Western blot analysis with anti-HA.11 (1:1000) (**a**) or silver staining (**b**) was then performed.

4.1.3 GLUT4 yield was increased using suspension adapted HA-GLUT4-GFP

HeLa cells

In order to generate a larger yield of GLUT4, HeLa HA-GLUT4-GFP adherent cells were adapted to suspension culture in order to easily grow a larger quantity of cells. This was achieved by gradually reducing the amount of serum (FBS), and then transferring them into suspension flasks with suspension media. This allowed for scaling up to about 3×10^7 cells per purification. HA-GLUT4-GFP from suspension adapted cells was shown to retain its insulin response using the surface GLUT4 assay described in chapter 3, showing that they remain a good model system for studying GLUT4 (**Figure 4-5**). It was also demonstrated later that PNGase treatment of GLUT4 resulted in the same molecular weight size shift, suggesting that the *N*-glycan has not been significantly altered through the process of adaption (**Figure 4-14a**).

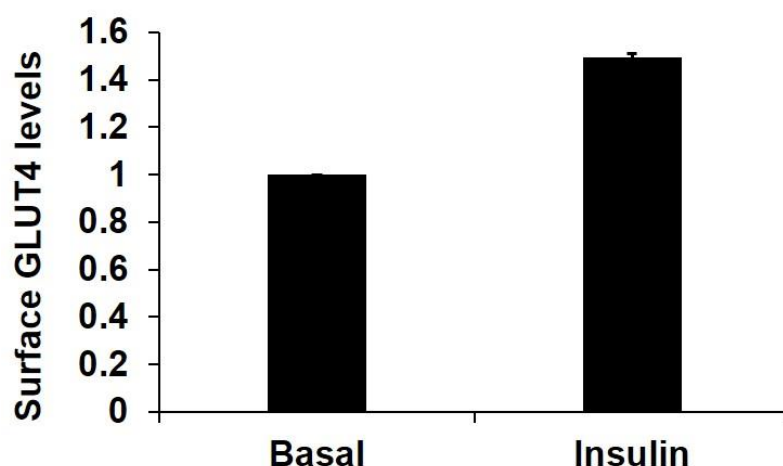


Figure 4-5 - Suspension HeLa cells respond to insulin

a) HA-GLUT4-GFP HeLa suspension cells were serum starved with serum free DMEM for 2 hours, and insulin ($1\mu\text{g/mL}$) was added to cells at 90 minutes. Cells were fixed in 2% PFA, then surface stained with α -HA antibody (1:1000) and α -mouse Alexa 647 (1:500). HA:GFP ratio was measured by flow cytometry to show the proportion of GLUT4 present at the surface. Data is normalised to basal cells, $n=2$

Using large amounts of suspension adapted cells resulted in much higher yields of GLUT4 as shown by both western blot and silver stain (**Figure 4-6**). The silver stain also showed that GLUT4 had a good level of purity (**Figure 4-6b**). The exact amount was quantified by measuring the fluorescent GFP signal of the sample and comparing it to GFP standards, giving an average concentration of around 5-10 $\mu\text{g/mL}$ per purification. This is sufficient for monosaccharide composition analysis, which uses around 2-3 μg per repeat.

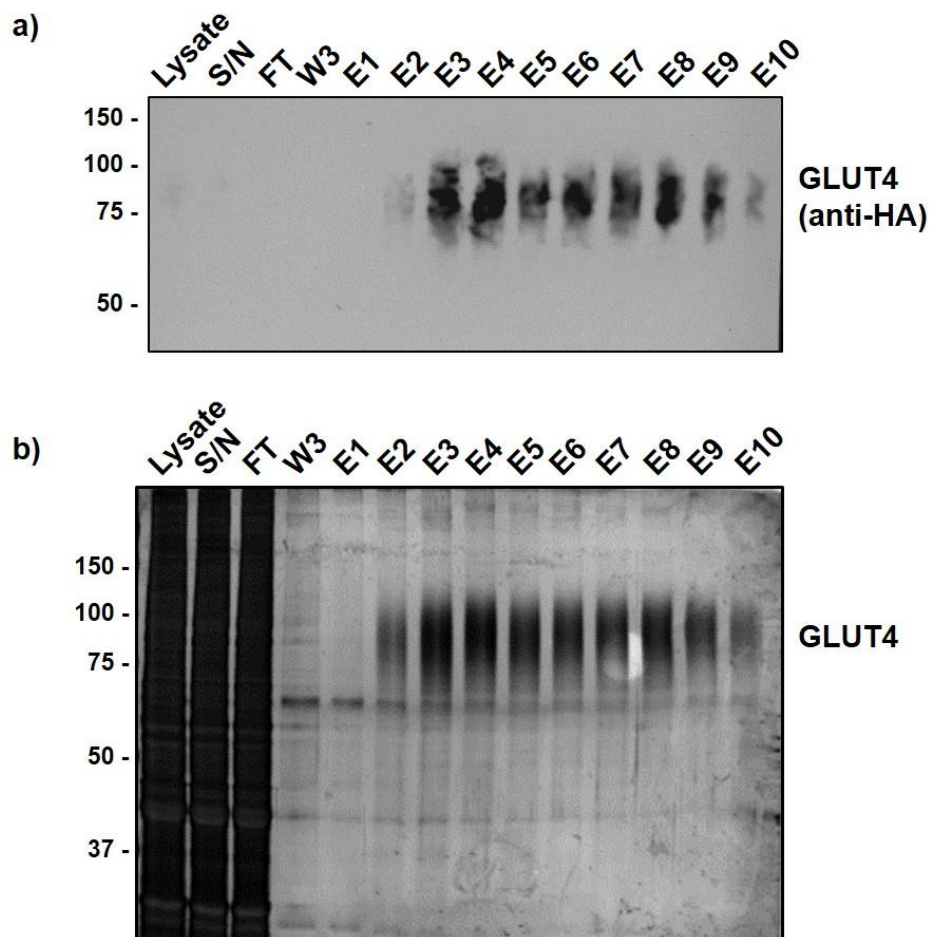


Figure 4-6 – GLUT4 purification with suspension cells produces higher yield
 GLUT4 was purified from HA-GLUT4-GFP HeLa suspension cells using a GFP-nanotrap. Eluates were collected in 10 collection tubes (E1-10), and run on 10% SDS-PAGE gel. Samples were run on 10% SDS-PAGE gel: Lysate, Supernatant (S/N), Flow-through (FT), wash 3 (W3) and elutions (E2-9). Western blot analysis with anti-HA.11 (1:1000) (**a**) or silver staining (**b**) was then performed.

4.1.4 Derivatised monosaccharide standards are visualised using HPLC coupled to a fluorescence detector

Once successful purification of GLUT4 was achieved, development of a monosaccharide composition analysis method by HPLC was performed, based on a protocol by Saddic *et al*, 2008. Monosaccharides are released from the purified protein through acid hydrolysis and fluorescently labelled with either anthranilic acid (AA) for aldose sugars, or O-phenyldiamine (OPD) for the α -ketose sialic acid. Both labels reductively react with the monosaccharide to form a covalent bond (**Figure 4-7**) and have been found to be the best labels for aldose and ketoses respectively (Anumula, 1994; Anumula, 1995; Anumula, 2006).

Following labelling, the sample is injected into a reverse phase HPLC column. The individual monosaccharides bind to the column with different affinities based on their hydrophobicity, and are released with a solvent which gradually increases in hydrophobicity. The monosaccharides, which are separated by time, pass through a fluorescence detector, and the observed peaks can be quantified relative to known standards. The entire schematic for aldose sugars is shown in **Figure 4-8a**, including the re-N-acetylation of amino sugars using acetic anhydride. Re-N-acetylation was performed on some samples as hydrolysis conditions removes the N-acetyl group on GlcNAc and GalNAc. The de-acetylated form of these monosaccharides elute too early for detection, preventing their quantification.

Using this method, separation and visualisation of the typical monosaccharide standards was achieved – specifically Galactose (Gal), Mannose (Man), Glucose (Glc), Fucose (Fuc), N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) (**Figure 4-8b**). These analytes were sufficiently separated into distinct peaks to facilitate quantification.

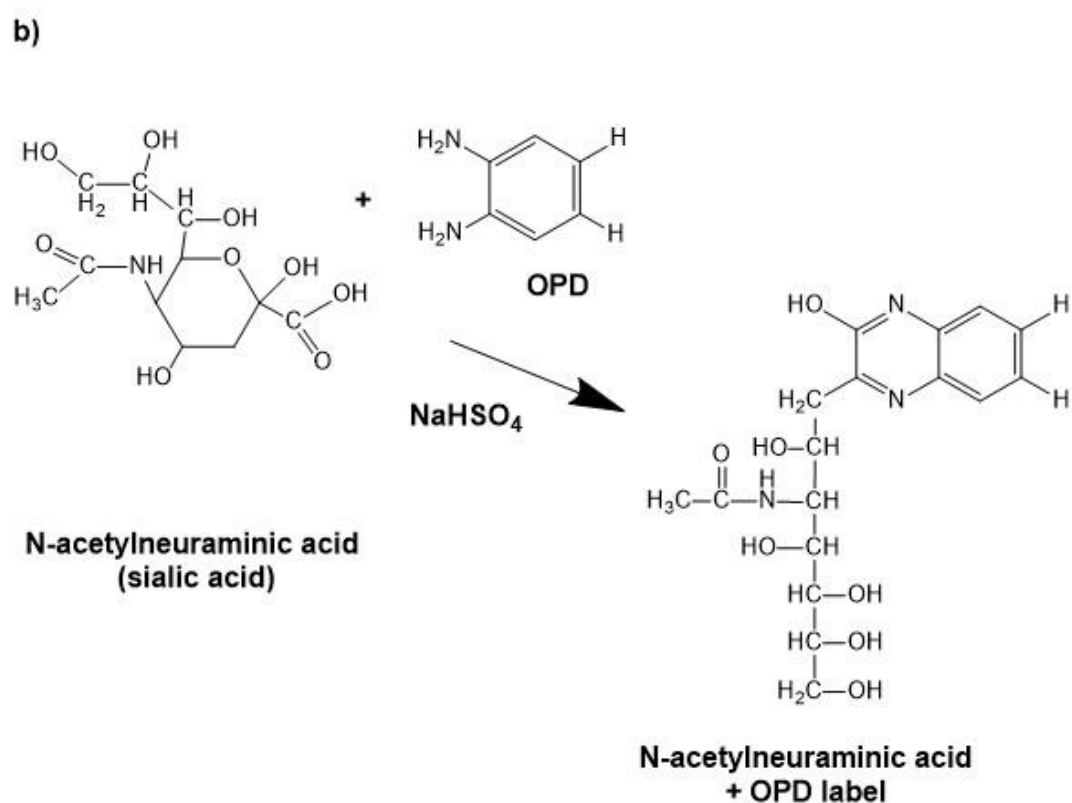
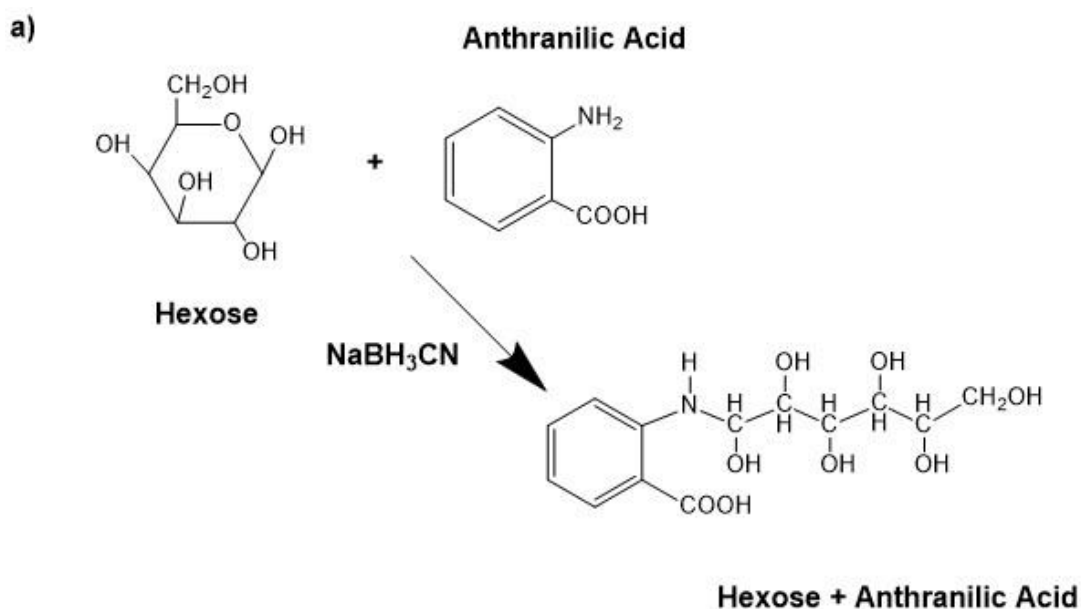


Figure 4-7- Fluorescent labelling reactions

a) Labelling reaction for aldose sugars with anthranilic acid, using NaBH_3CN in the reductive reaction. **b)** Labelling reaction for ketose sugar with O-Phenyldiamine (OPD) using NaHSO_4 in the reductive reaction

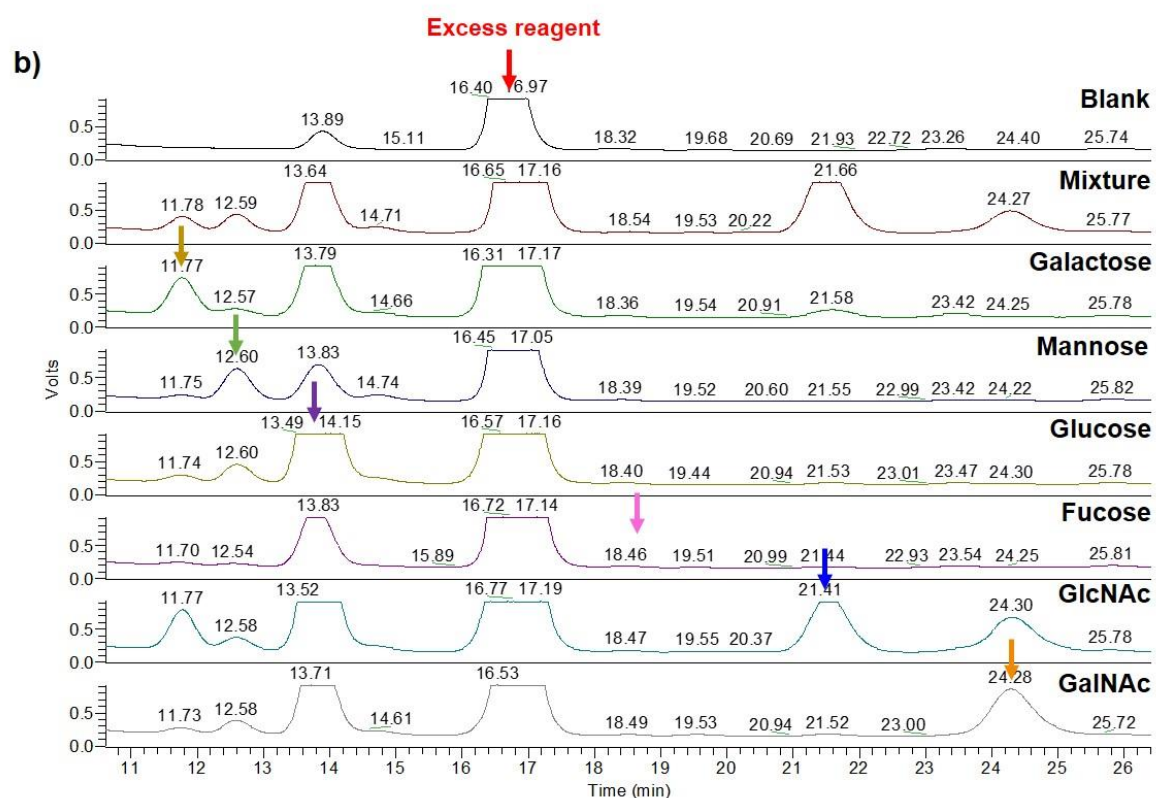
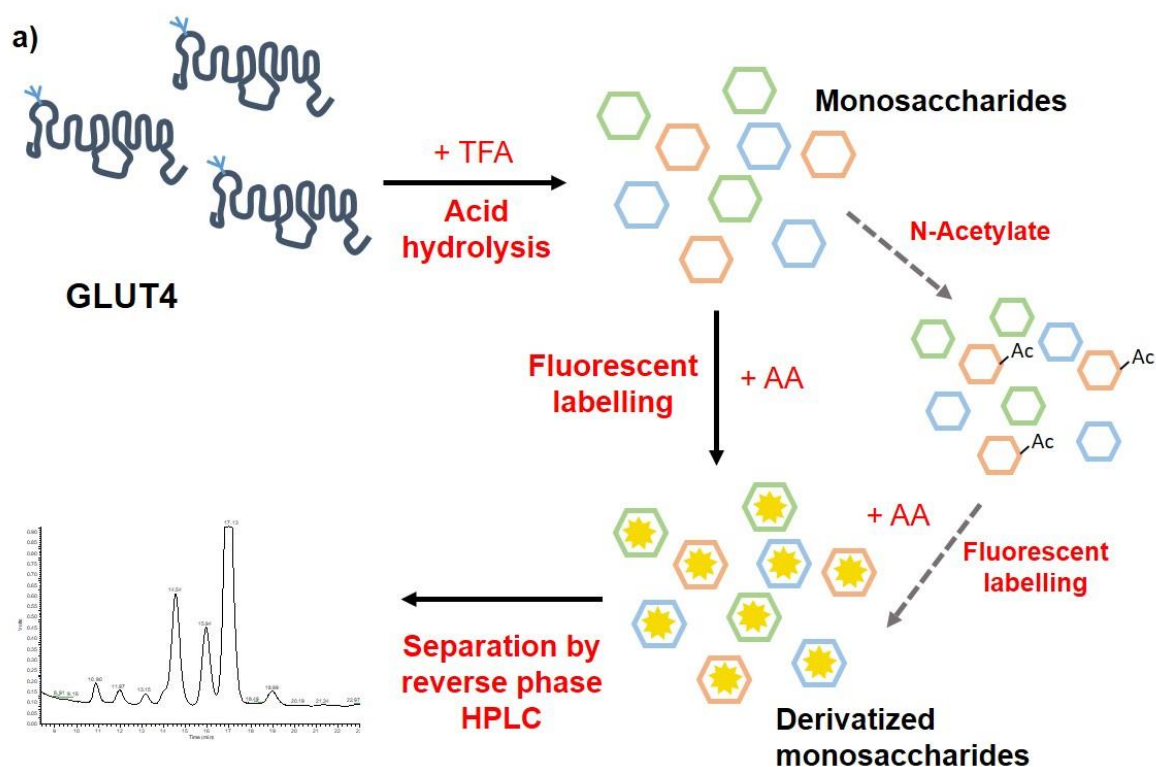


Figure 4-8 – Monosaccharide composition analysis separates peaks for monosaccharide standards

a) Flow diagram schematic for preparing aldose sugars released from purified glycoprotein for reverse-phase HPLC **b)** Reverse phase HPLC was carried out on 25 pmol monosaccharide standards derivatized with anthranilic acid. GlcNAc and GalNAc were hydrolysed and acetylated before derivatisation. Mixtures contains all the monosaccharide standards, at 25 pmol each.

4.1.5 Optimisation of monosaccharide composition analysis with Fetuin

Following the identification of monosaccharide standard peaks, optimisation of monosaccharide composition analysis on a glycoprotein was required. GLUT4 sample was limited, so this was performed on fetuin, a commercially available glycoprotein with multiple *N*-glycan sites (Lin et al., 2018) (**Figure 4-9**). This showed that hydrolysis was successful at releasing monosaccharides from the protein and they were sufficiently separated for the quantification of individual peaks (**Figure 4-9**). The amounts and ratios observed were consistent with previous data (Yuan et al., 2005), although GlcNAc was not observed as N-acetylation was not performed on this sample.

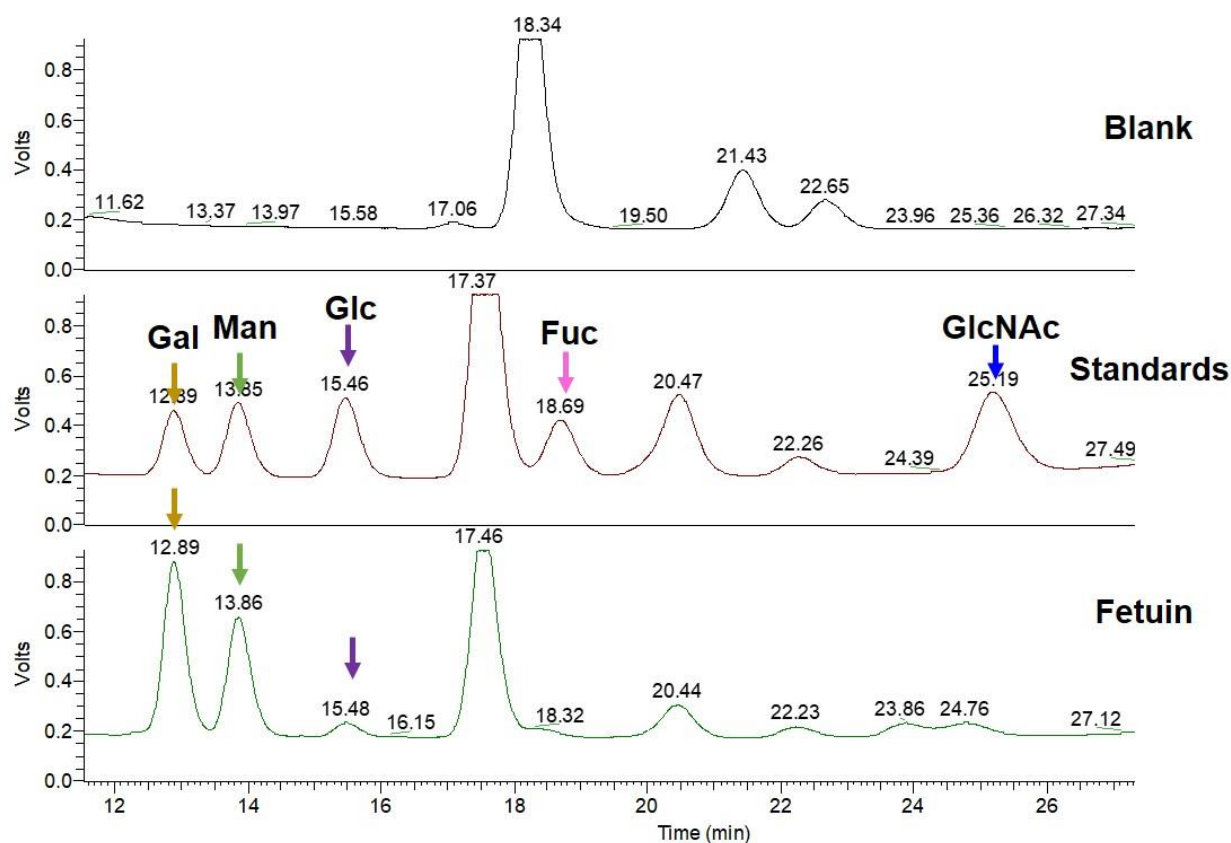


Figure 4-9 - Monosaccharide composition analysis on Fetuin

5 μ g Fetuin and monosaccharide standards (25 pmol) were hydrolysed and derivatized with anthranilic acid, then injected through a reverse phase column

To test whether the elution buffer used for GLUT4 purification affected the visualisation of monosaccharide peaks, fetuin was resuspended in 0.1 M glycine, pH 2.7. A small amount of LMNG was also added to address concerns that residual amounts of the dimeric glucose moiety present in the detergent would interfere with monosaccharide quantification. Rather than performing acid hydrolysis directly on the protein sample, the sample was dried onto membrane (PVDF or nylon) and washed in ddH₂O to try and remove any excess glucose. Hydrolysis was then performed on the minced membrane before derivatisation and separation as before.

Glucose peaks (indicated by the purple arrow) were observed in all chromatograms except the blank at around 17.7-18.5 minutes, along with an excess reagent peak at 19-21 minutes (**Figure 4-10a-e**). This indicates that washing the membrane was not sufficient to remove glucose. The presence of glucose peaks in the blank membranes also indicates that they themselves are a source of contamination. It was therefore decided that performing hydrolysis directly on the protein sample is preferable rather than drying onto membrane first, to avoid the risk of additional contamination.

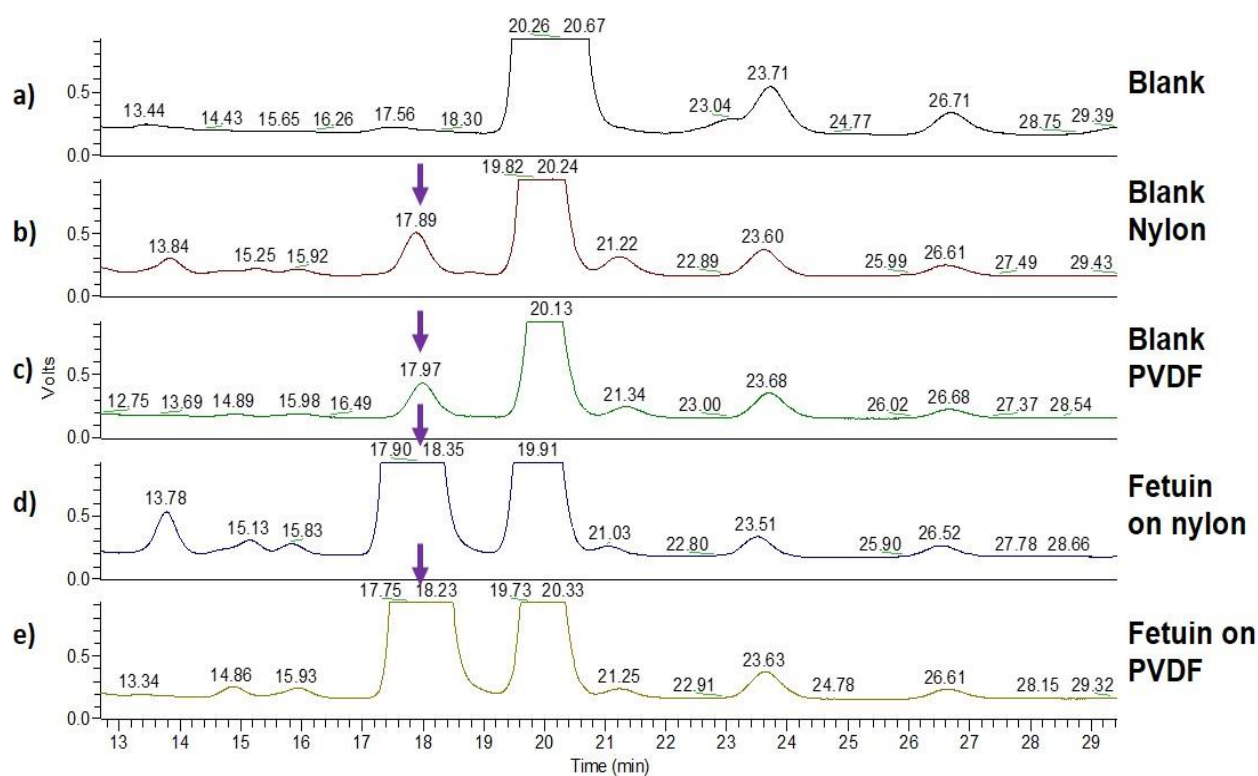


Figure 4-10 – Monosaccharide composition of Fetuin using membranes

5 μ g of lyophilised Fetuin was dried onto nylon (d) or PVDF (e) membrane. Along with blank nylon (b) and PVDF (c), these were washed with water and hydrolysed in TFA. They were then derivatized with AA, and reverse phase HPLC was performed, along with a blank with just buffer (no membrane) (a).

4.1.6 Monosaccharide composition analysis of GLUT4's *N*-glycan revealed polylactosamine structure

Using the optimised method including N-acetylation (**Figure 4-8a**), monosaccharide composition analysis was performed on GLUT4 and monosaccharide standards (**Figure 4-11a**). Although there was the expected large glucose peak in the GLUT4 sample, this was small enough to avoid interfering with the other peaks, so quantification was possible. Extra glucose was also observed in the standard mixture, as it can also come from the environment. Given that glucose is unlikely to be present in the *N*-glycan anyway, all glucose observed in the GLUT4 sample can be assumed to either come from LMNG or environmental contamination.

Chromatography peaks are observed for galactose, mannose and GlcNAc. Fucose was not observed in either the standard mixture or GLUT4 sample for this run, but low levels of fucose are observed in other runs (**Figure 4-13**).

In addition to aldose analysis, sialic acid analysis was also performed. Purified GLUT4 was hydrolysed with milder conditions to limit sialic acid degradation, then derivatized with OPD. This sample was then subjected to reverse phase HPLC with fluorescence detection with the same solvent conditions as the aldose analysis (**Figure 4-11b**). Multiple sialic acid peaks were observed in both the sialic acid standard sample and the GLUT4 sample (indicated with the red arrow), suggesting that some degradation may still be present (**Figure 4-11b**).

Quantification of all the peaks was achieved by comparing the area of the GLUT4 monosaccharide peaks to the standards prepared from a known amount of

monosaccharide. Previous data has indicated that the *N*-glycan is in the complex form, so it can be assumed that 3 mannose monomers are present per glycan (**Figure 3-1 & Figure 3-4**). Therefore, quantification of galactose, fucose, GlcNAc and sialic acid was measured relative to 3 mannose monomers, as shown in **Figure 4-12**. Estimates showed that there are roughly 10 galactose monomers present and 9 GlcNAc monomers. The expectation based on known complex *N*-glycan structures is that there are normally two more GlcNAc monomers than galactose. However, visualisation of GlcNAc is dependent on N-acetylation of the sample, of which it is difficult to assess the efficiency, so it is possible that not all the GlcNAc was acetylated. If this is the case, the ratio is roughly consistent with the presence of an *N*-acetyllactosamine (LacNAc) dimer. In addition, the quantities of galactose and GlcNAc indicate that there are multiple LacNAc dimers present, demonstrating the presence of a polylactosamine chain. This confirms the hypothesis that the large size of GLUT4's *N*-glycan is at least partly attributed to the presence of polylactosamine.

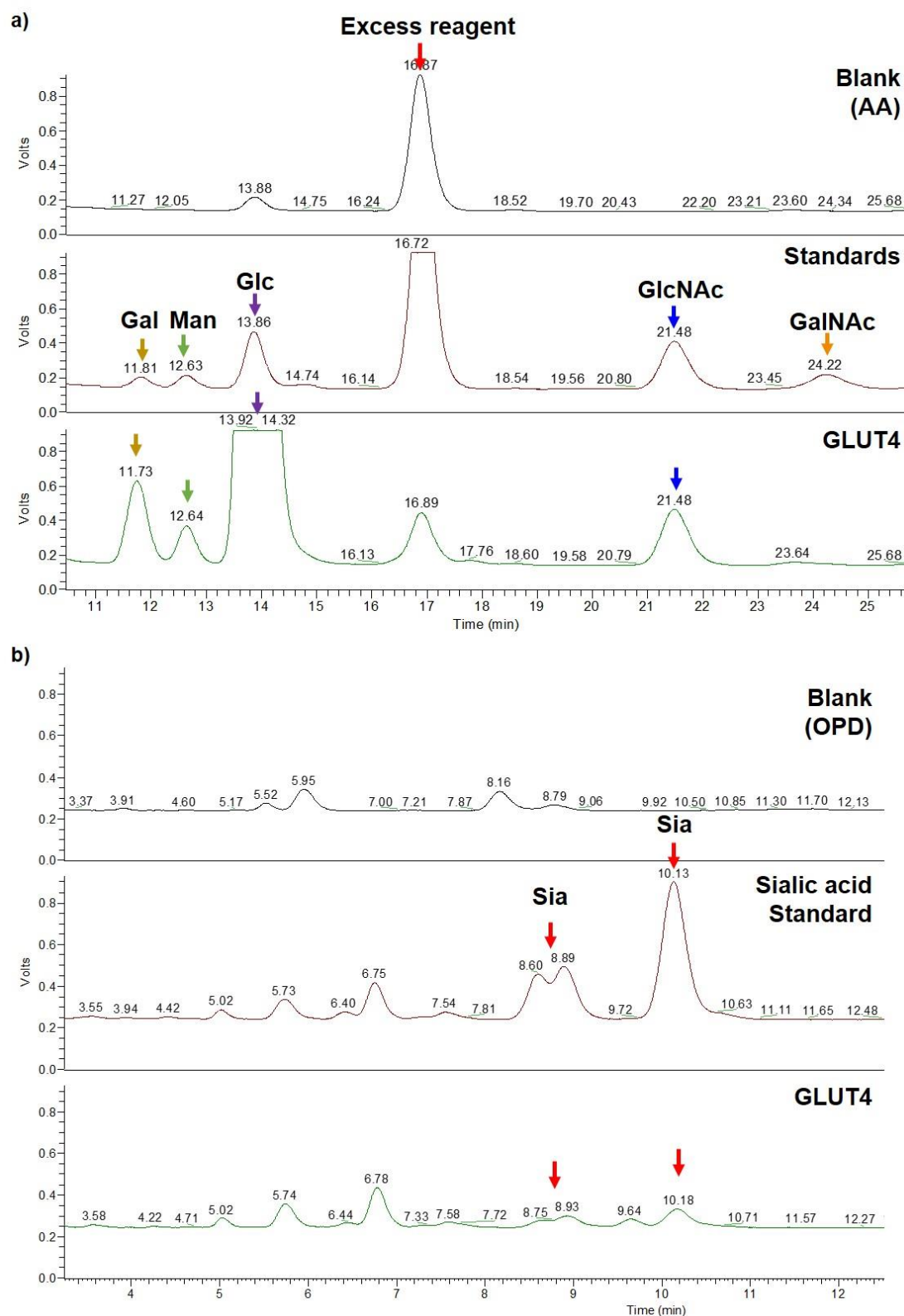


Figure 4-11 - Monosaccharide composition analysis of GLUT4 of aldoses and sialic acid
a) Purified GLUT4 and monosaccharide standards (25 pmol) were hydrolysed, acetylated and derivatized with AA, then injected through a reverse phase column. **b)** Purified GLUT4 and sialic acid standard (100 pmol) were mildly hydrolysed and derivatized with OPD, then injected through a reverse phase column. Both were run alongside blanks containing just the buffers/labelling reagent used.

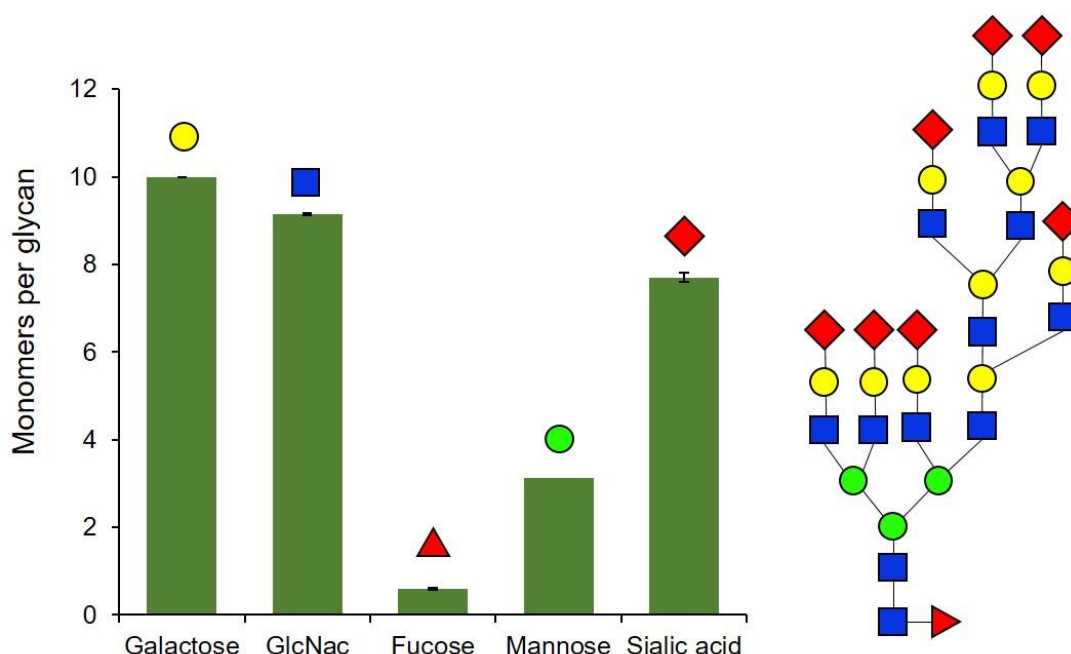


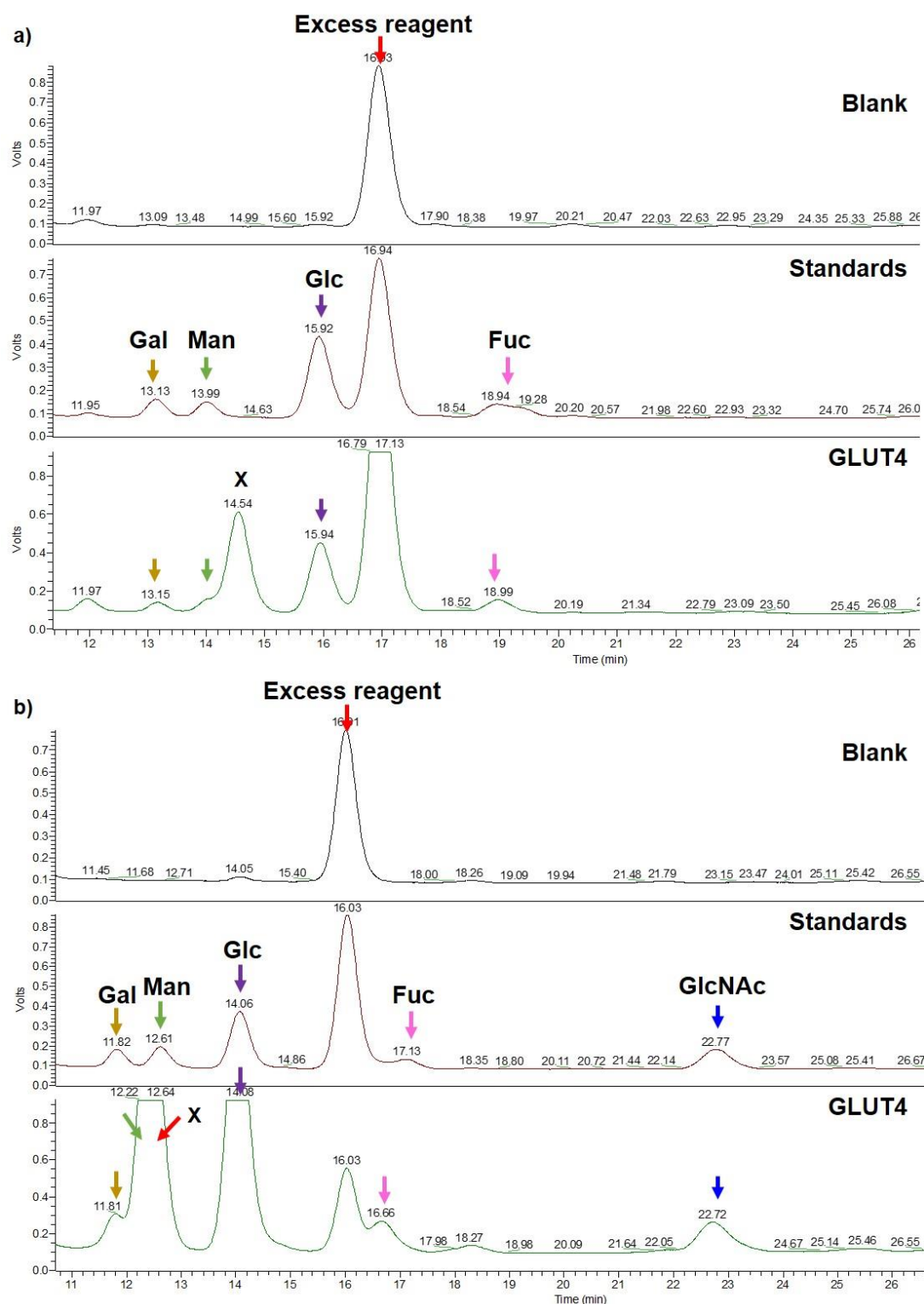
Figure 4-12 - Quantification of monosaccharide composition analysis peaks

Peak areas of the monosaccharide standards were compared with the GLUT4 peaks, quantifying the mmole/mg protein of each monosaccharide and normalising the quantities relative to 3 mannose subunits (the number of mannose monomers present in a complex glycan). A model structure based on this data was then made.

Based on the quantification of monosaccharide monomers, an approximate model of the *N*-glycan was produced (**Figure 4-12**). While the exact number of antennae is uncertain, the number of sialic acid residues present suggest that the polylactosamine chain is branched. This is because sialic acid is found on terminal galactose monomers, and a linear polylactosamine chain would not provide enough terminal ends for the number of sialic acid monomers (estimated to be 7 or 8).

This approximate *N*-glycan structure has an estimated size of ~7.5 KDa. However, GLUT4's *N*-glycan is predicted to be around 25-35 kDa based on the size change of GLUT4 with PNGase F treatment (**Figure 3-2**). This suggests that there is a component of the *N*-glycan which has not been accounted for.

When analysing the chromatograms from the monosaccharide composition analysis, a large peak was observed across multiple repeats in both hydrolysed (**Figure 4-13a**) and acetylated (**Figure 4-13b**) GLUT4 samples – labelled peak X. This peak overlaps with mannose, but **Figure 4-13a** shows that it is a distinct peak. Peak X is seen consistently across 5 different GLUT4 preps, but not in the standards or blanks, nor was it observed in fetuin (**Figure 4-10**). Its absence in **Figure 4-11a** was fortunate as it allowed the quantification of mannose, but quantification of the glucose:galactose ratio compared to **Figure 4-13** suggests that it simply shifted into the glucose peak. This is possibly due to variations in the chromatography conditions, such as small changes in the composition of the solvents. Peak X could therefore account for the missing component of the *N*-glycan, especially given that the peak is visibly significantly larger than galactose.



4.1.7 Peak X is found in the *N*-glycan fraction

Before attempting to identify peak X, it was first important to determine whether the peak was coming from the *N*-glycan itself or somewhere else on the protein, given that all the monosaccharides commonly found in *N*-glycans have been accounted for. The *N*-glycan was separated from GLUT4 with a 30 kDa filter following treatment with PNGase F. Subjecting samples to western blot analysis revealed a significant decrease in the molecular weight of GLUT4, indicating that PNGase F treatment was effective (**Figure 4-14a**). The absence of a band in the *N*-glycan sample also indicates that GLUT4 was effectively excluded from that fraction (**Figure 4-14a**).

Following hydrolysis and derivatisation with anthranilic acid, fluorescence coupled HPLC revealed that peak X was present in the *N*-glycan fraction and not the GLUT4 fraction (**Figure 4-14b**). This shows that peak X is a part of the single *N*-glycan, rather than coming from elsewhere on GLUT4. Identifying peak X is therefore crucial to obtaining an accurate structure model of the *N*-glycan.

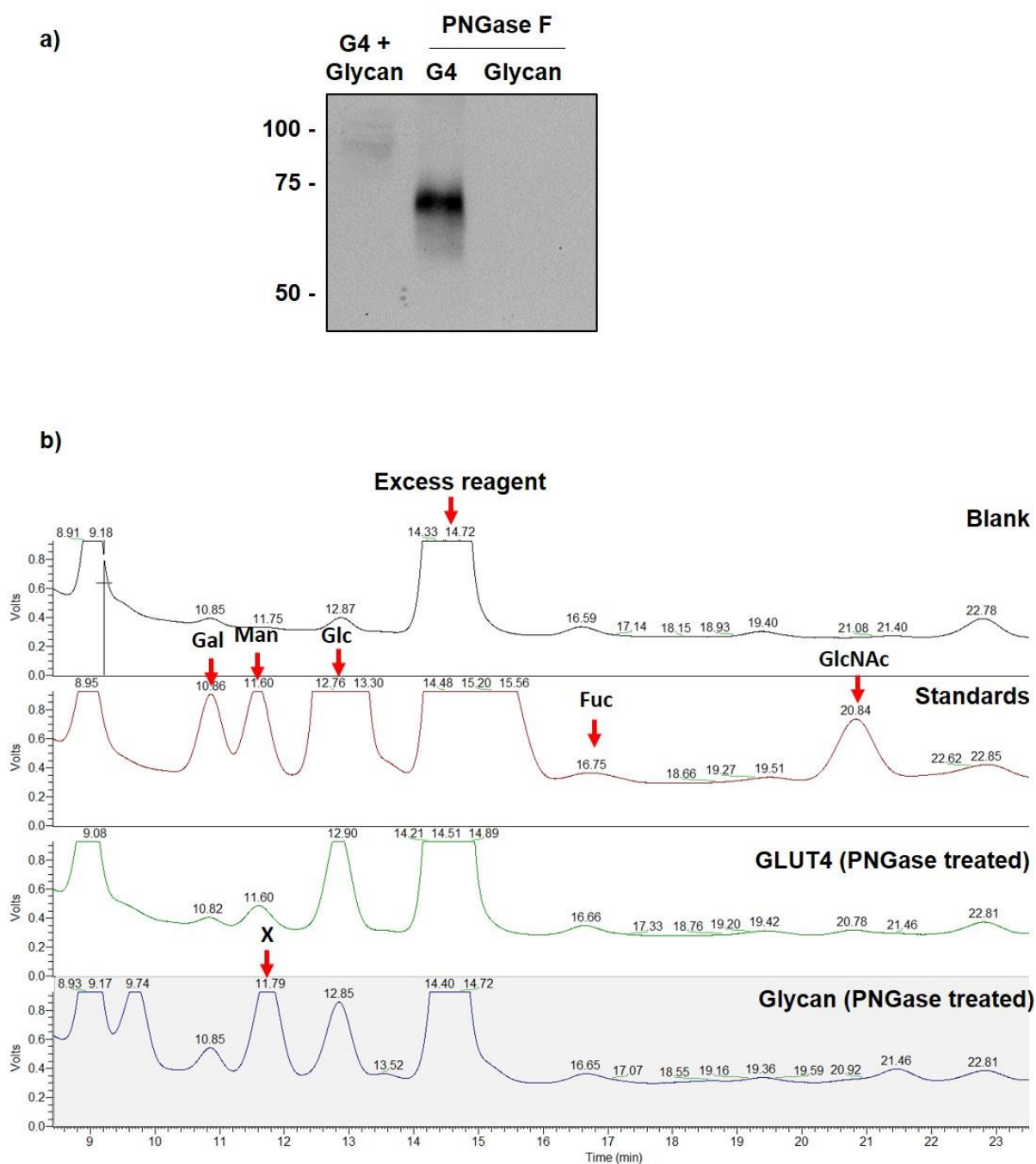


Figure 4-14 – Monosaccharide composition analysis of separated GLUT4 and N-glycan fractions

a) Purified GLUT4 (G4) was denatured and treated with PNGase F overnight. GLUT4 was then separated from the cleaved N-glycan using a 30 kDa filter unit. Samples were then run alongside untreated GLUT4 on a 10% SDS-PAGE and subjected to western blot analysis with anti-HA.11 (1:1000). **b)** PNGase treated and separated GLUT4 and N-glycan were hydrolysed, acetylated and derivatized alongside monosaccharide standards, then injected into a reverse phase HPLC column.

4.1.8 Identification of peak X

To try and identify peak X, HPLC with fluorescence detection was performed on monosaccharide standards that have not previously been found in mammalian *N*-glycans. These standards were xylose, rhamnose and arabinose (**Figure 4-15**). However, neither the xylose nor rhamnose peaks overlapped with mannose (where peak X normally appears) showing that these were not the right candidates. Arabinose was not detected at all, which is possibly due to it being eluted too early for detection, which indicates that this isn't the right compound either.

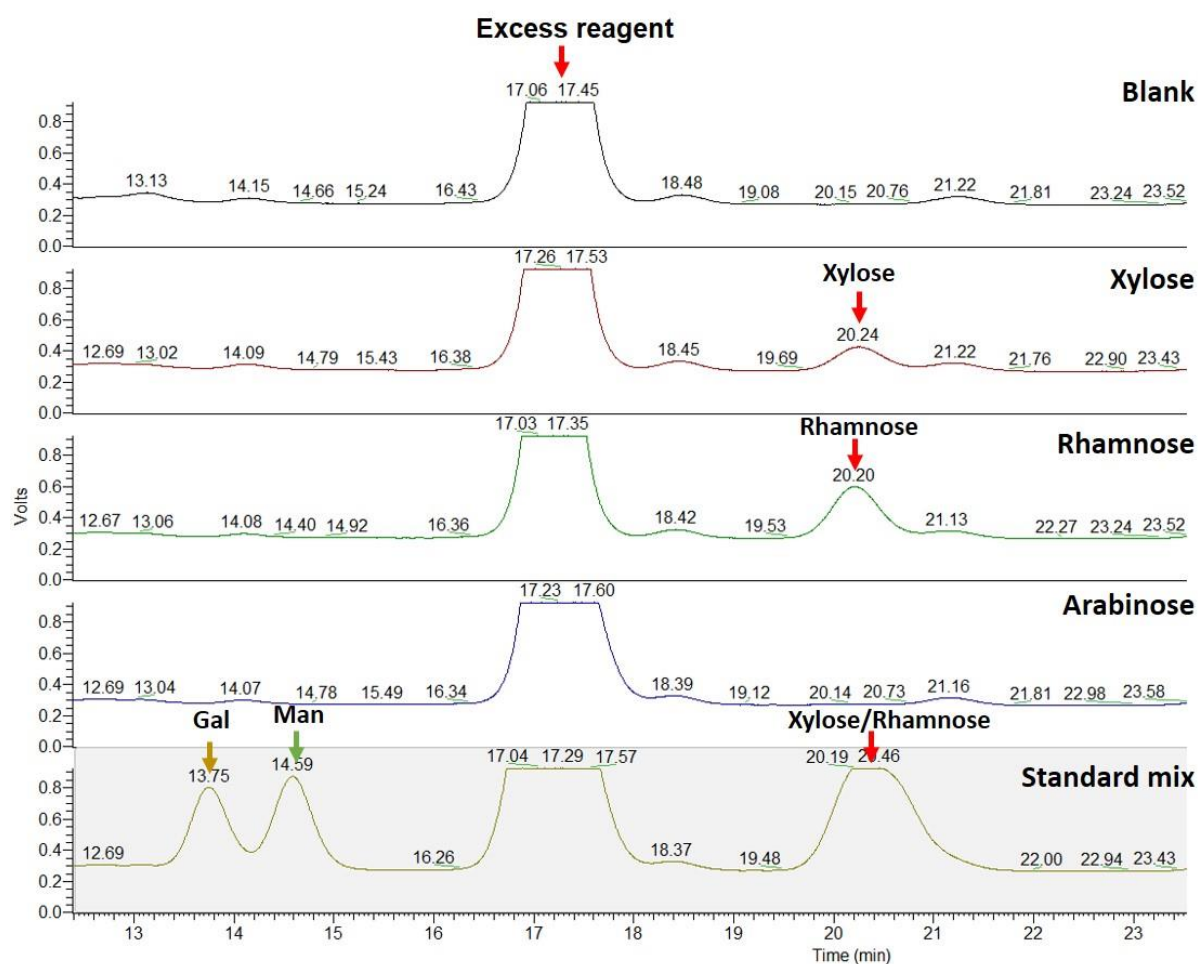


Figure 4-15 – Reverse phase HPLC on unusual monosaccharide standards

25 pmol Xylose, Rhamnose or arabinose were derivatized alongside 25 pmol monosaccharide standard mixture of galactose, mannose, xylose, Rhamnose and arabinose. Samples were then separated by fluorescent-linked reverse phase HPLC.

To use a more direct approach to identifying the unknown monosaccharide, fractions of peak X were collected from both hydrolysed and N-acetylated GLUT4 samples after reverse phase separation. Blanks were collected where no peak was seen, and the galactose peak was also collected. Whole samples without fractionation were also analysed. Peak X did not move upon acetylation, so its identity was likely a compound found in both hydrolysed (Hid) peak X and acetylated (N-Ac) peak X fractions, but not in the blank or galactose fractions.

Electrospray ionisation LC-MS was performed on the fractions which produced a list of m/z values along with a matching chemical formula (**Table 4-1**). The abundance of each compound are reported for each fraction, calculated from the areas under each ion intensity peak.

Many of the compounds produced could be ruled out without further investigation. For example, while compound 2 has the biggest abundance in the fractions of interest, it is not a formula which can produce a biologically possible structure based on the possible number of covalent bonds for each element. In addition, the hydrolysis process likely removes any phosphate groups, so it is unlikely for the compound of interest to contain a phosphorous, ruling out compounds 2-5. Despite its low presence in the acetylated sample, as visualised by the ion intensity graph, the most likely candidate is compound 1, $C_{11}H_{16}N_2O_4$ (**Figure 4-16a**). No peaks are observed for blank or galactose fractions, showing its presence is specific to the peak X fraction. Compound 6 is possibly an acetylated version of compound 1, which is consistent with its absence in the whole hydrolysed sample and its presence in the whole acetylated sample. Given that peak X is observed in both hydrolysed and re-acetylated samples, further investigation is needed to confirm the identity of peak X.

However, in the absence of another viable candidate, $C_{11}H_{16}N_2O_4$ remains the main compound of interest. Removing the elements present in the anthranilic acid label produces a formula of $C_4H_9NO_3$ which likely represents an amino tetrose such as erythrosamine or threosamine (structures shown in **Figure 4-16b**).

Table 4-1 - Compounds identified through LC-MS performing on HPLC fractions (performed by Swen Langer from the Centre of Excellence in Mass Spectrometry).

Group areas are recorded in each fraction and colour-coded. Green = present (area > 50,000), yellow = low presence (area > 5000), red = absent (area < 5000). Hyd = fractions from hydrolysed GLUT4 without acetylation, N-Ac = fractions from hydrolysed GLUT4 with acetylation, Whole = hydrolysed GLUT4 sample without fractionation. Blank = fraction from with no visible peak (or buffer only for Whole), Gal = galactose fraction, peak x = peak x fraction.

	Formula	Formula without label (-C7H7NO)	Calc. MW	Hyd, Blank	Hyd, Gal	Hyd, Peak X	N-Ac, Blank	N-Ac, Gal	N-Ac, Peak X	Whole, Hyd	Whole, Blank	Whole, N-Ac
1	C11H16N2O4	C4H9NO3	240.1	1,500	1,903	122,915	2,009	2,024	7,557	20,728,201	15,726	2,691,557
2	C13H29N6O5 P	C6H22N5O4 P	380.2	2,829	2,012	605,495	2,336	14,172	7,079,146	4,155	3,794	3,199
3	C14H25N10O P	C7H18N9P	380.2	3,337	2,965	305,855	3,296	10,328	3,564,595	8,643	11,791	7,523
4	C9H23N6O4P	C2H16N5O3 P	310.15	2,551	2,315	594,399	2,278	2,271	235,239	4,277	3,967	3,266
5	C14H29N6O5 P	C7H22N5O4 P	392.2	2,741	2,037	4,136	2,160	2,171	1,093,736	8,828	2,953	4,148
6	C13 H18 N2 O5	C6H11NO4	282.1	2,611	1,926	1,426	2,986	2,759	6,048	4,056	3,491	3,418,937

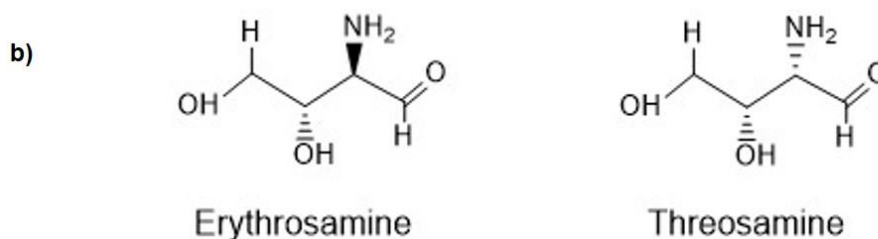
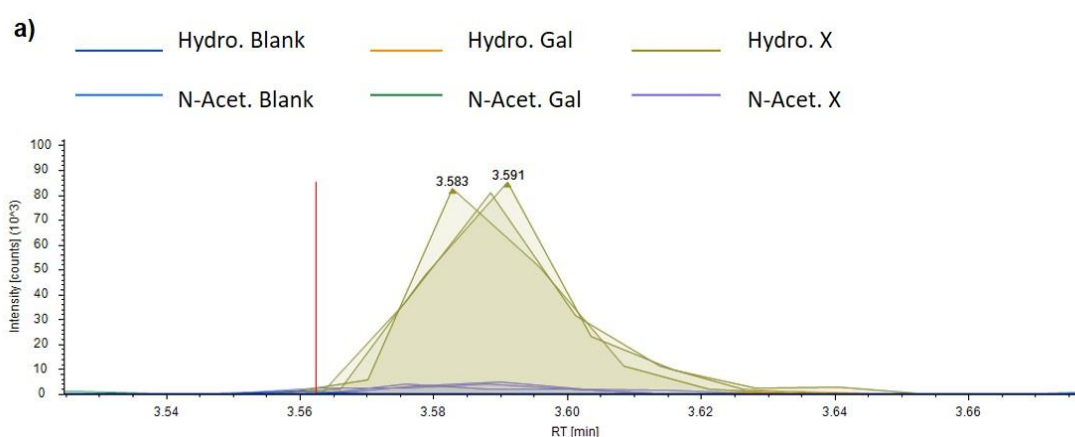


Figure 4-16 – Abundance of C11H16N2O4 from different HPLC fractions

a) ESI LC-MS was performed on Blank, galactose (gal) and peak X (X) fractions, from both hydrolysed and acetylated samples. 3 technical replicates of each fraction were performed. Data collected by Swen Langer from the Centre of Excellence in Mass Spectrometry **b)** Chemical structures of erythrosamine and threosamine, which are potential candidates for peak x

4.2 Discussion

This chapter reports the elucidation of a partial structure for GLUT4's *N*-glycan. A complex *N*-glycan with a branched polylactosamine chain was identified. In addition, there was evidence for the presence of a unique monosaccharide that has not previously been reported in mammalian cells.

A method for purifying HA-GLUT4-GFP from HeLa cells was first optimised by combining two previous studies to produce a large enough pure sample of GLUT4 (Kraft et al., 2015; Rothbauer et al., 2008). Monosaccharide composition analysis using fluorescent labelling was then used to detect monosaccharide peaks separated using reverse-phase HPLC (Saddic et al., 2008).

While the presence of polylactosamine in GLUT4's *N*-glycan had been speculated based on the molecular weight shift of GLUT4 when the *N*-glycan is removed, quantification of the monosaccharide composition now provides evidence in support of this. The presence of polysialic acid was ruled out, as this is primarily found in the nervous or immune systems (Colley et al., 2014; Villanueva-Cabello et al., 2021). In addition, polysialic acid has a much greater degree of polymerisation with sizes ranging from 50-400 residues, whereas only 7-8 sialic acid residues were found on GLUT4's *N*-glycan (Nakata and Troy, 2005). The amount of sialic acid present therefore indicates that the polylactosamine is branched, as it is typically found on the terminal ends of the branches.

The formation of linear polylactosamine requires the alternate action of β 1-4 galactosyltransferases and β 1-3-*N*-acetylglucosaminyltransferases. Branches are formed by the action of β 1-6-*N*-acetylglucosaminyltransferases onto internal galactose residues. This

specific branching class of enzymes could therefore be targeted through knockdown experiments, forcing a linear polylactosamine structure. This could provide further evidence for the branched structure of polylactosamine, and the importance of branched vs linear structure to GLUT4's *N*-glycan function.

The presence of polylactosamine, while unsurprising, is significant. The composition of an *N*-glycan determines the proteins capable of binding, and the degree of affinity. Galectins are a family of proteins which bind to N-acetylactosamine dimers, and their affinity increases as length and branching of the polylactosamine chain is increased (Hirabayashi et al., 2002). The identification of a polylactosamine chain means that the galectins now represent a protein family of interest in respect to GLUT4 binding, and will be explored further in the following chapter.

In addition to polylactosamine, a large part of the *N*-glycan was also found to contain a monosaccharide that as yet has not been identified in mammalian cells. This was observed by the presence of a large peak (peak X) in the GLUT4 sample. This peak was not present in the blank, the standard mixture, or in fetuin, so it did not come from contaminants in the hydrolysis or derivatisation process. This monosaccharide was confirmed to be a part of the *N*-glycan by separating GLUT4 and its *N*-glycan into different fractions.

Compounds identified by LC-MS as being present in the peak X fraction but not galactose or blank fractions produced a list of potential candidates. However, many of these candidates could be excluded due to the biological impossibility of their structure. For example, each carbon can make a maximum of 4 covalent bonds, but some of the formula would require more than that to form a structure which incorporates all of the elements. Compounds containing phosphorous could also be ruled out, as the hydrolysis reaction would remove a

phosphate group and it is unlikely for a phosphorous to be present outside of a phosphate group. This indicates that the LC-MS produced a lot of false positives, which may be reduced if it was performed on sample containing cleaved *N*-glycan only, as this would be a purer sample. This would be a priority for future experiments.

The most abundant compound found in the hydrolysed sample is the $C_{11}H_{16}N_2O_4$, which when the label is removed gives the formula $C_4H_9NO_3$ – representing an amino tetrose such as erythrosamine or threosamine. Although it is not well characterised, $C_4H_9NO_3$ is a structure found in biology in the form of erythrosamine, so this represents the most likely candidate. However, it is not found in the acetylated fraction in a significant quantity and an acetylated version ($C_6H_{11}NO_4$) is found in the whole (unfractionated) sample. Peak X appears at the same position in the acetylated sample, so it was expected that the compound does not contain an acetyl group, and would not be able to be acetylated. This suggests that either the amino tetrose does not make up the bulk of peak X, or another species moves into the same position. The latter would indicate that peak X actually represents two different monosaccharides in the hydrolysed and acetylated samples.

In addition, amino tetrose is 119 kDa, and although it is hard to quantify peak X given its overlap with mannose, a rough estimation based on the size of the peak is that the structure would contain around 70 amino tetrose residues. There may be elements of the structure that were removed in the hydrolysis step, which would increase the molecular weight and therefore reduce the number of residues slightly. However, this still represents a very large proportion of the structure, raising the question of where these residues would go, and whether they exist in a polymer form.

Although further investigation is needed to answer some of the questions surrounding its presence/absence in different fractions and the large quantity found in the structure, the amino tetrose is the best candidate that has been identified so far. A biologically known tetrose is erythrose, which is an intermediate in the pentose phosphate pathway, although the amine version has not previously been identified in mammalian cells. However, 2-amino-2-deoxy-d-erythrose (or d-erythrosamine) has previously been identified in the cell wall of the common mushroom *Agaricus bisporus* showing that it exists in biology (Fujii and Maruoka, 1982).

There is some precedent to a novel metabolite being uniquely found in the glycan of one protein. Ribitol-5-phosphate is an alditol that in mammalian cells has been uniquely identified on α -dystroglycan, and plays a key role in the function of the protein (Kanagawa et al., 2016; Praissman et al., 2016). This metabolite had previously been found exclusively in bacteria and plants, and at the point of its discovery, the enzyme pathway for its synthesis was not known. It is possible that erythrosamine (or threosamine) could be another such monosaccharide, uniquely present on GLUT4.

Confirming that an amino tetrose elutes at the same point as peak X in HPLC would be key evidence to support its presence on the *N*-glycan. However, amino tetroses (such as erythrosamine) are not commercially available. To produce a monosaccharide standard, it would possibly need to be synthesised from scratch, which would be challenging. A simpler method could be pursued through the reduction of 2-amino-2-deoxy-D-erythronic acid (which is available commercially) to generate the erythrosamine standard. This would enable the direct comparison of erythrosamine with peak X. Further analysis could include

treatment of the *N*-glycan with endo-galactosidase to cleave polylactosamine from the *N*-glycan prior to HPLC analysis to elucidate where the compound is located on the structure.

In addition to identifying peak X, there is still more analysis that needs to be done to elucidate the full structure of GLUT4's *N*-glycan – for example identifying the linkages between monosaccharides. However, the findings in this chapter have significantly progressed the knowledge of GLUT4's *N*-glycan by identifying and quantifying the monosaccharides present, and in particular confirming the presence of polylactosamine and the degree of branching.

5 Analysing the role of galectins in mediating

GLUT4's *N*-glycan function

Data presented in Chapter 3 support a role for GLUT4's *N*-glycan in the intracellular sequestration of GLUT4 under basal conditions. Previous studies have demonstrated that GLUT4 undergoes both clathrin-mediated and clathrin-independent endocytosis (CME and CIE respectively) but many questions regarding GLUT4's internalisation remain (Blot and McGraw, 2006; Nishimura et al., 1993; Ros-Baro et al., 2001). For example, while the CIE of GLUT4 has been shown to be cholesterol-dependent, the exact mediators of this process have not been defined (Ros-Baro et al., 2001).

One mechanism of CIE occurs through uncoated membrane invaginations called clathrin-independent carriers (CLICs), which can form through the binding of galectins to glycoproteins on the cell surface, a process which has been named the GL-Lect model (Johannes et al., 2016; Lakshminarayan et al., 2014). Galectins are a family of carbohydrate binding proteins that bind to β -galactoside sugars such as N-acetyllactosamine, with a preference for branched polylactosamine (Hirabayashi et al., 2002). The finding that GLUT4's *N*-glycan contains branched polylactosamine (chapter 4), led to the hypothesis that the role of GLUT4's *N*-glycan in intracellular sequestration is through galectin-mediated endocytosis.

In support of this hypothesis, elevated serum galectin-3 levels are associated with type 2 diabetes (Weber et al., 2009; Weigert et al., 2010; Yilmaz et al., 2015), and galectin-1 levels are elevated in adipose tissue of people living with type 2 diabetes (Fryk et al., 2016).

Furthermore, addition of galectin-3 to 3T3-L1 adipocytes has been shown to reduce the insulin-stimulated increase of surface GLUT4 (Li et al., 2016). These observations are consistent with a role for galectin family members in regulation of whole body glucose homeostasis.

In contrast to the GL-Lect model, galectin-glycoprotein binding has also been shown to form a lattice preventing glycoprotein internalisation (Markowska et al., 2011; Partridge et al., 2004). A model combining these two contrasting functions proposes that galectin binding acts along a spectrum; as the concentration of galectin increases, galectin action switches from promoting endocytic pit entry to forming a galectin-glycoprotein lattice with a cell surface retention role (Mathew and Donaldson, 2019). While this has largely been observed for galectin-3, there has been some evidence of other galectins (such as galectin-1) regulating surface expression of glycoproteins (Belardi et al., 2012).

N-glycan structures with more branching have a greater avidity for galectins (Hirabayashi et al., 2002). Artificially increasing the degree of glycoprotein branching has been shown to increase surface GLUT4 expression, suggesting that galectin binding can cause the retention of GLUT4 (Lau et al., 2007). However, this doesn't exclude the possibility that galectins normally promote GLUT4 endocytosis, and that by increasing galectin binding, the mode of action is switched into a retention role.

The aim of this chapter is to investigate the role of galectin family members as mediators of GLUT4 endocytosis. A variety of methods are used in order to provide a comprehensive analysis on the physiological role that galectins play.

5.1 Results

5.1.1 Inhibition of galectin binding causes an increase in surface GLUT4

Galectins binding to their *N*-glycan ligands can be globally inhibited through the addition of excess lactose to the cells (Lakshminarayan et al., 2014). As shown in **Figure 5-1a**, lactose is a β -galactoside disaccharide of galactose and glucose which can be bound by galectins, and can outcompete binding to *N*-glycans when added in excess. Other disaccharides such as sucrose (fructose-glucose) or maltose (glucose-glucose) are not β -galactoside sugars so are not thought to be ligands for galectin binding, providing negative controls.

Addition of sugar solutions to serum free growth medium (DMEM) increases its osmolarity. Increased osmolarity is one of the stimuli through which GLUT4 is translocated to the cell surface, as it mimics the effects of muscle contraction in exercise (Brozinick et al., 1994; Li et al., 2001). To control for this effect, sugar solutions were diluted to the same osmolarity as DMEM only (~0.3 Osm). The osmolarity of diluted sugar solutions was found to be the same as DMEM only, so any effects observed with the sugar solutions can't be attributed to increased osmolarity (**Figure 5-1b**).

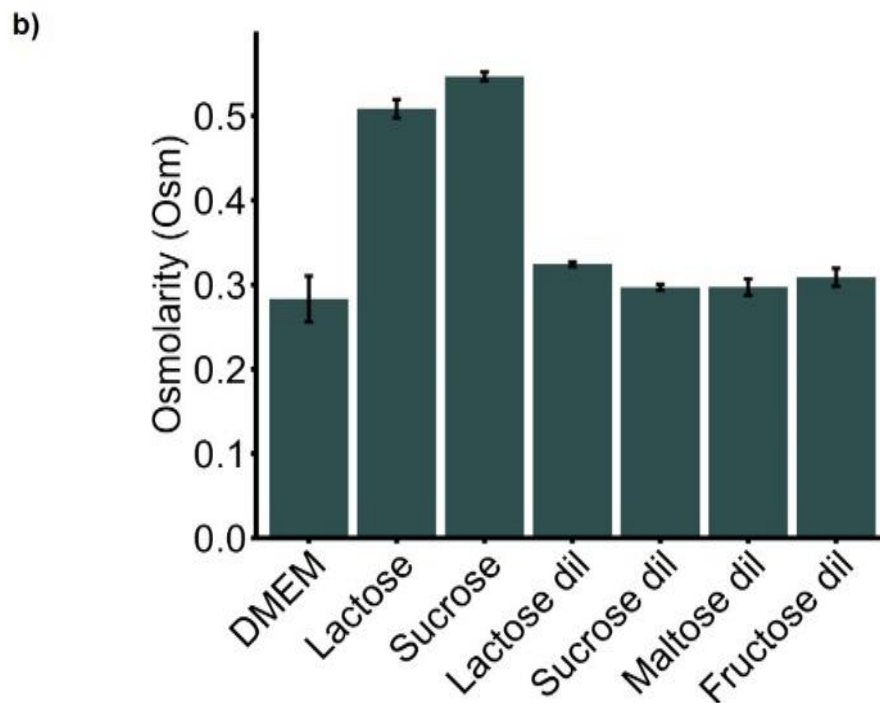
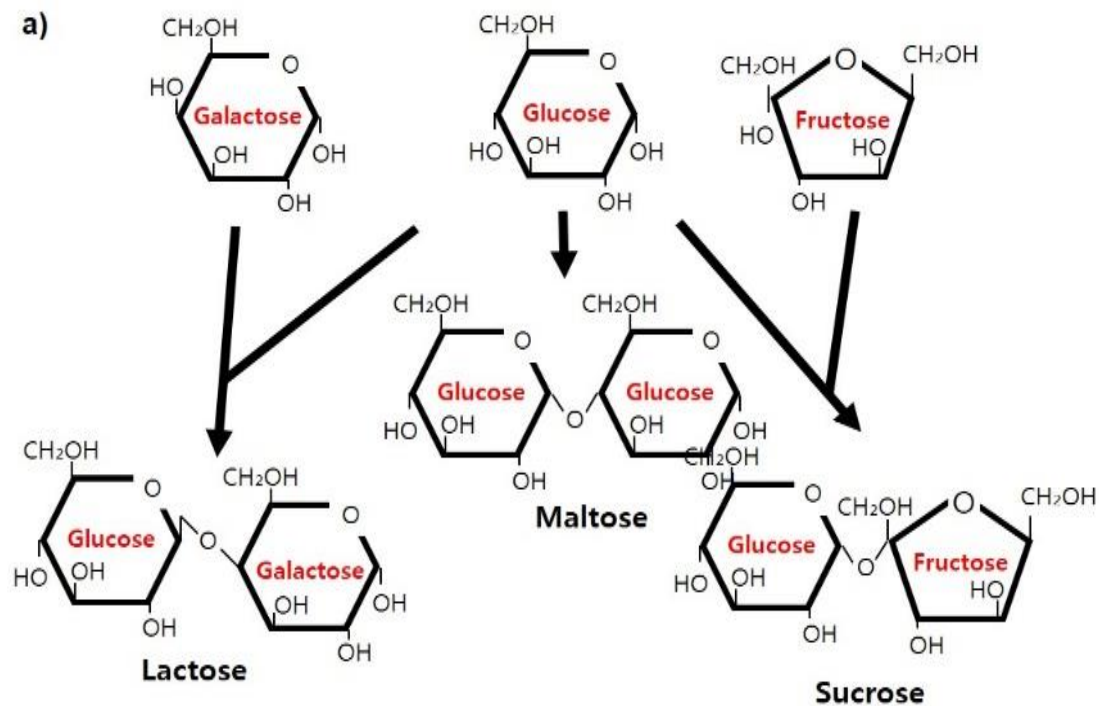


Figure 5-1 – Osmolarity of sugar solutions

a) Monosaccharide structures for galactose, glucose and fructose and disaccharide structures for lactose, maltose and sucrose **b)** Osmolarity of solutions was measured using an osmometer. 200mM solutions of lactose or sucrose in serum free DMEM were made up, and compared to 200mM solutions of lactose, sucrose, maltose and fructose in serum free DMEM diluted 1:3, n=3

These diluted sugar solutions were used to investigate whether galectins are involved in the regulation of GLUT4 trafficking. Using the flow cytometry assay described in section 3.1.2, surface GLUT4 levels were measured following addition of sugar solutions to HA-GLUT4-GFP HeLa cells. **Figure 5-2a** shows that addition of lactose to the medium results in increased surface GLUT4, an effect not seen in the presence of sucrose. This observation is consistent with the hypothesis that galectin binding is involved in promoting GLUT4 internalisation (**Figure 5-2a**). Contrary to expectations, this increase was also seen for maltose (a glucose-glucose disaccharide), which would not be expected to bind galectins as it not a β -galactoside sugar (**Figure 5-2a**). The monosaccharides that make up these disaccharides (glucose, galactose and fructose) along with glucosamine were investigated to try and observe a pattern for which monosaccharide was causing an effect, but these also showed no change in surface GLUT4. It is possible that the glucose residues in maltose are in a similar enough conformation to lactose that when added in excess it can prevent galectin binding, but this may also indicate that maltose blocks binding of a different protein interactor.

Previous data demonstrated that the *N*-glycosylation inhibitor kifunensine causes a significant increase in surface GLUT4 in basal conditions (section 3.1.2). Kifunensine treatment was performed in the presence of lactose and sucrose to determine whether the effects of lactose on surface levels of GLUT4 are mediated through *N*-glycosylation. Lactose addition and kifunensine treatment alone both cause a significant increase in cell surface GLUT4 levels (**Figure 5-2b**). Combination of the two treatments does not have an additive effect, suggesting that they are acting through the same pathway. In contrast, sucrose treatment does not cause an increase in surface GLUT4, and a significant increase is still

observed in sucrose + kifunensine treatment compared to sucrose only. Collectively the data presented in **Figure 5-2b** indicate that the effect of lactose on the cell surface levels of GLUT4 is mediated through disruption of galectin-glycan interactions.

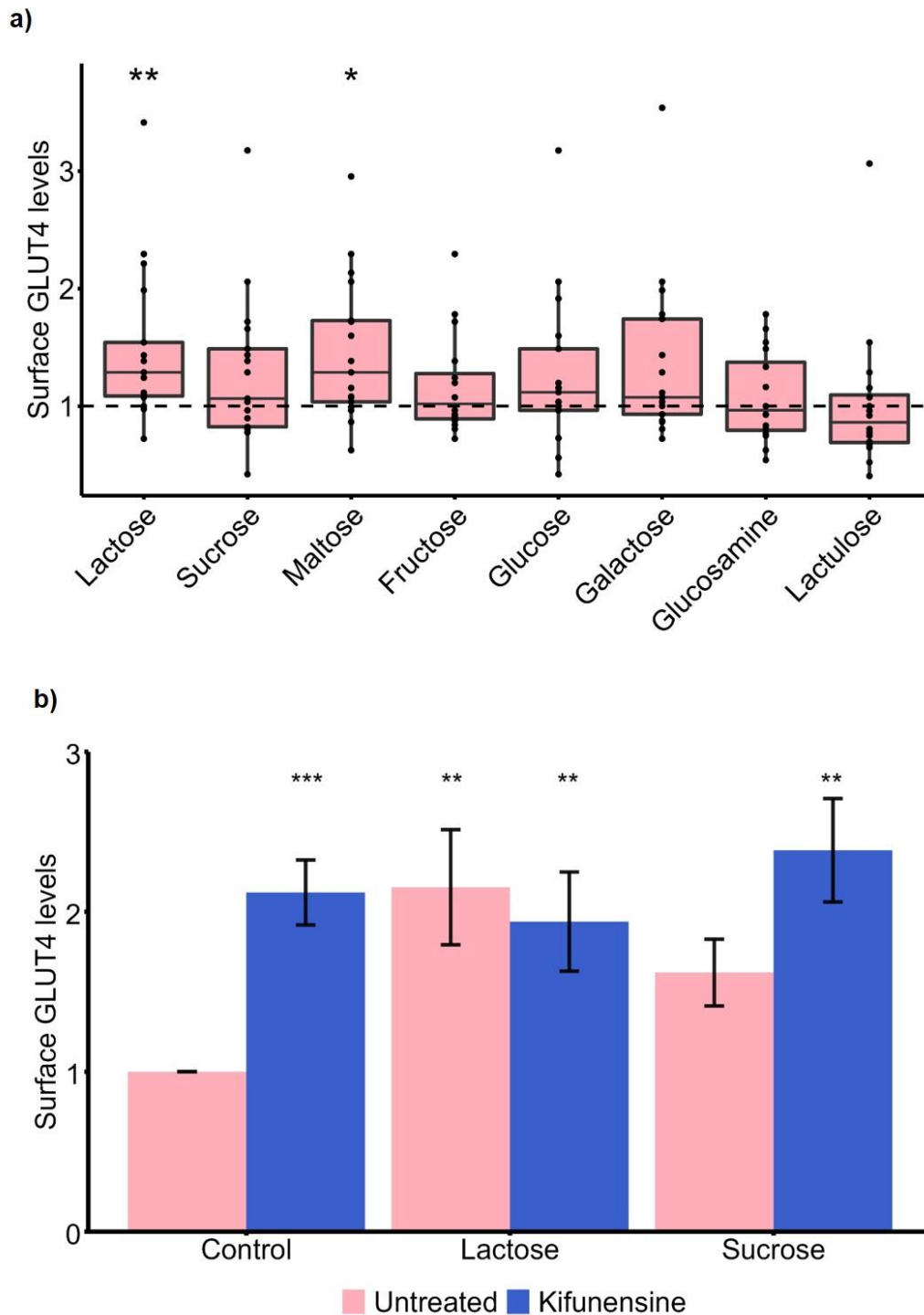


Figure 5-2 - Effect of sugars on surface GLUT4

a) HA-GLUT4-GFP expressing HeLa cells were treated with sugar solutions. These sugars were dissolved in serum-free medium diluted to the same osmolality as DMEM only. The cells were serum starved with the sugar solutions for 2 hours, and then surface stained with α -HA antibody (1:1000) and α -mouse Alexa 647 (1:500) to label the GLUT4 present at the cell surface at the moment of fixation. HA:GFP ratio was measured by flow cytometry to show the proportion of GLUT4 present at the surface. Dotted line represents medium only, against which all data was normalised against. * = $p < 0.5$, ** = $p < 0.1$ compared to medium only **b)** Kifunensine was added to HA-GLUT4-GFP HeLa cells at $2 \mu\text{g/mL}$ at time of cell plating and incubated overnight, before performing surface GLUT4 assay as described. All data is normalised to untreated control, $n=6$ ** = $p < 0.01$ compared to untreated control

5.1.2 Lactose pulldown assay reveals galectin-1 and -3 are present in HeLa cells

As a first step to identifying which galectin(s) are involved in regulating the levels of GLUT4 present at the cell surface, a lactose pulldown assay was performed to identify which galectins are present in HA-GLUT4-GFP HeLa cells by performing a lactose pulldown assay. Secreted galectins immediately bind to glycoproteins in their local environment rather than dispersing, so galectin concentration in the medium is likely to be low. In addition, growth medium contains fetal bovine serum (FBS) which may contain galectins, adding a source of contamination. For these reasons, the lactose pulldown assay was performed on cell lysates rather than conditioned media.

Cell lysates were incubated with lactose-agarose beads, and lactose binding proteins were then eluted with a high concentration of lactose (500 mM). Separation of protein samples by SDS-PAGE followed by silver stain revealed a single protein band with a molecular weight of around 12-13 kDa (**Figure 5-3a**). No protein band was seen bound to agarose-only beads, which was an important control as agarose is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose (**Figure 5-3b**). In addition, eluting the protein with sucrose did not remove the protein from lactose beads, further demonstrating its specificity to lactose (**Figure 5-3b**). MALDI-TOF mass spectrometry (performed by the Centre of Excellence in Mass Spectrometry) revealed this protein band to be galectin-1, validating it as candidate of interest for binding GLUT4's *N*-glycan.

Galectin-3 had previously been identified as a protein of interest based on its association with type 2 diabetes (Weber et al., 2009; Weigert et al., 2010). To see whether galectin-3

could be mediating GLUT4's *N*-glycan function, protein samples were subjected to western blot analysis with an anti-galectin-3 antibody (**Figure 5-3c**). This showed that galectin-3 is also expressed in HeLa cells and binds specifically to lactose. Its absence in the silver stained gel suggests it has lower intracellular levels, which may be reflective of an overall lower protein expression, or due to a higher efficiency of secretion.

In **Figure 5-2**, maltose was also found to increase surface GLUT4 levels, suggesting that it may be interfering with the binding of galectins or another interactor. A pulldown assay was performed with maltose beads, to observe whether there were proteins bands that bound maltose but not sucrose or fructose (**Appendix IV**). However, no bands were identified solely with maltose elution. Although further investigations into the maltose effect could reveal new aspects of *N*-glycan binding, this was not pursued further in this project, with the focus instead remaining on the galectins.

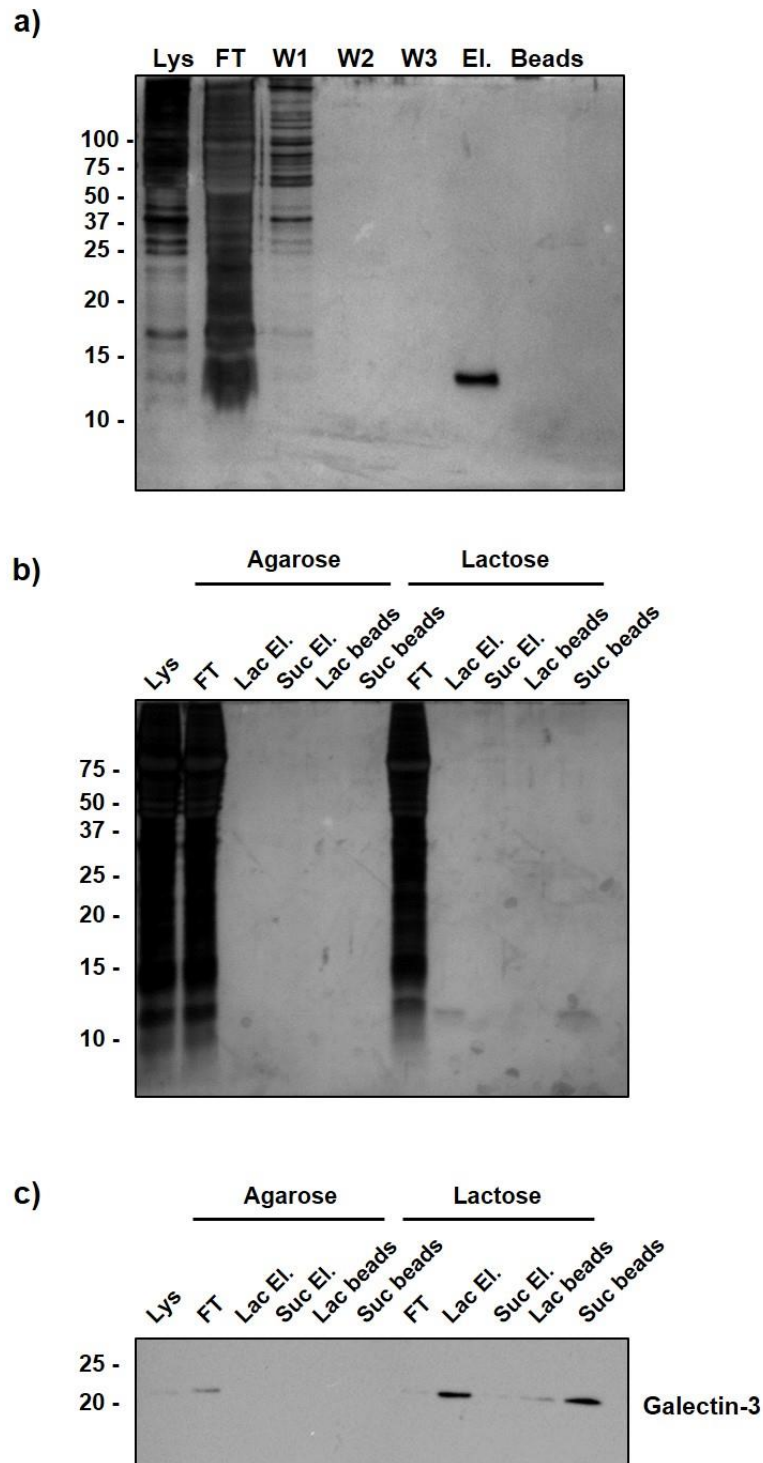


Figure 5-3 - Lactose pulldown assay

a) Lactose pulldown assay. HA-GLUT4-GFP Hela cells were incubated with lactose beads and washed to remove unbound proteins. Bound proteins were then eluted with lactose, and run on a 15% SDS-PAGE gel. This was then silver-stained in order to visualise the protein bands. **b)** Assay was repeated with both agarose only beads and lactose beads. Both lactose and sucrose were used for protein elution. **c)** Western blot was carried out with assay samples, and probed against galectin-3 (1:1000).

PL=protein ladder; Lys=lysate; FT = flow through; W1-3 = washes 1-3; El. = eluate.

5.1.3 Increasing galectin-1 & galectin-3 causes significant changes in surface

GLUT4

Having identified galectin-1 and galectin-3 as potential binding partners for GLUT4's *N*-glycan, the next step was to determine their effects on surface GLUT4. To this end, recombinant Gal1-His was purified from BL21 *E.coli* cells. This was run on an SDS-PAGE gel alongside recombinant Gal-3-His protein (previously purified by Hannah Black) and BSA standards to determine protein concentration (**Figure 5-4a**). This showed that galectin-1 concentration was 1.56 mg/mL and galectin-3 concentration was 5.8 mg/mL, and both proteins had a high level of purity.

To assess an appropriate concentration scale for adding additional galectin to the cells, the amount of galectin-1 and -3 already present at the cell surface was analysed. Increasing concentrations of each galectin were added to cells, and the fluorescent signal from galectin-bound antibody was measured relative to 0 μ M (**Figure 5-4b&c**). For galectin-1, the addition of 3 μ M gave a 10x signal increase relative to 0 μ M, so intervals of 0.5 μ M up to 3 μ M is a suitable concentration range (**Figure 5-4b**). For galectin-3, the increase in signal reached a plateau at 1 μ M with 1.8x signal increase relative to 0 μ M (**Figure 5-4c**). This plateau may be a result of extracellular galectin-3 binding sites being saturated, causing excess galectin-3 to be washed away. The concentration range of 0-1.5 μ M was therefore chosen, with 0.25 μ M intervals.

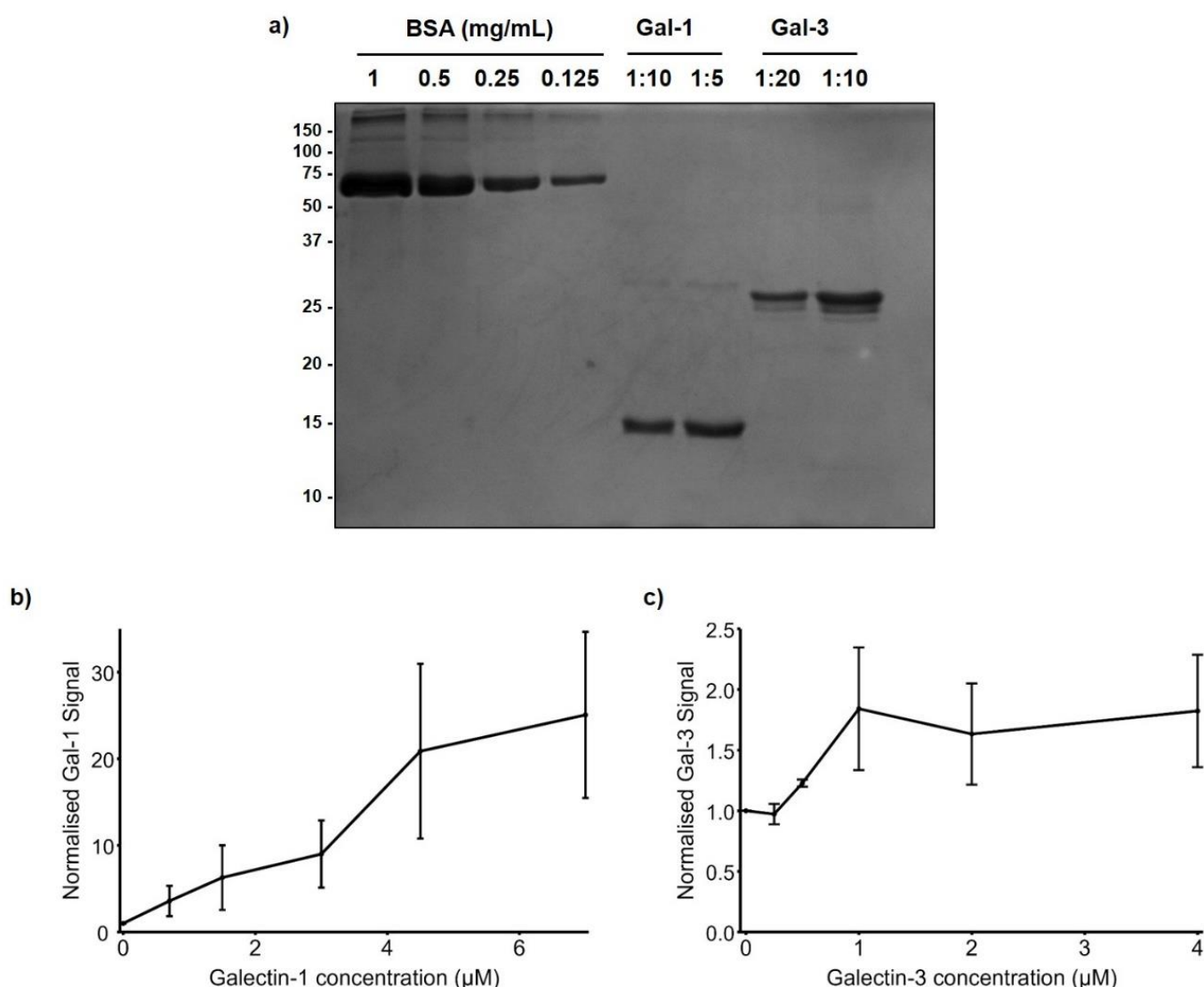


Figure 5-4 – Measuring surface galectin levels with recombinant galectin-1 and -3

a) Purified Gal-1 (diluted 1:10 and 1:5) and Gal-3 (1:20 and 1:10) were run on an SDS-PAGE gel alongside decreasing concentrations of BSA. Gel was Coomassie stained, and ImageJ was used to determine band intensity to estimate protein concentration. Galectin-1 concentration = 1.56 mg/mL, galectin-3 concentration = 5.8 mg/mL

b&c) HA-GLUT4-GFP cells were serum starved with serum-free DMEM for 2 hours, in the presence of increasing concentrations of (b) recombinant galectin-1 (0 µM, 0.7 µM, 1.5 µM, 3 µM, 4.5 µM, 7 µM) or (c) recombinant galectin-3 (0, 0.25 µM, 0.5 µM, 1 µM, 2 µM, 4 µM). Cells were fixed with 2% PFA, and then surface stained with anti-galectin-1 or -3 antibody (1:1000), and anti-mouse AlexaFluor 647 (1:500). Cells were then collected for flow cytometry. n=3

Having determined the appropriate concentration ranges for increasing extracellular galectin-1 and galectin-3, their effect on surface GLUT4 was analysed (**Figure 5-5**). Both galectins caused a significant but opposite change in surface GLUT4. A significant increase was observed when increasing galectin-1 concentration (**Figure 5-5a**), while a significant decrease was observed when increasing galectin-3 (**Figure 5-5b**). It is worth noting that these data show what each galectin will do with artificially high concentrations, not at physiological conditions. However, the galectin-3 data suggests it might mediate the role of GLUT4's *N*-glycan in intracellular sequestration identified in chapter 3, which is consistent with the observed effects of inhibiting galectin binding with lactose (**Figure 5-2**).

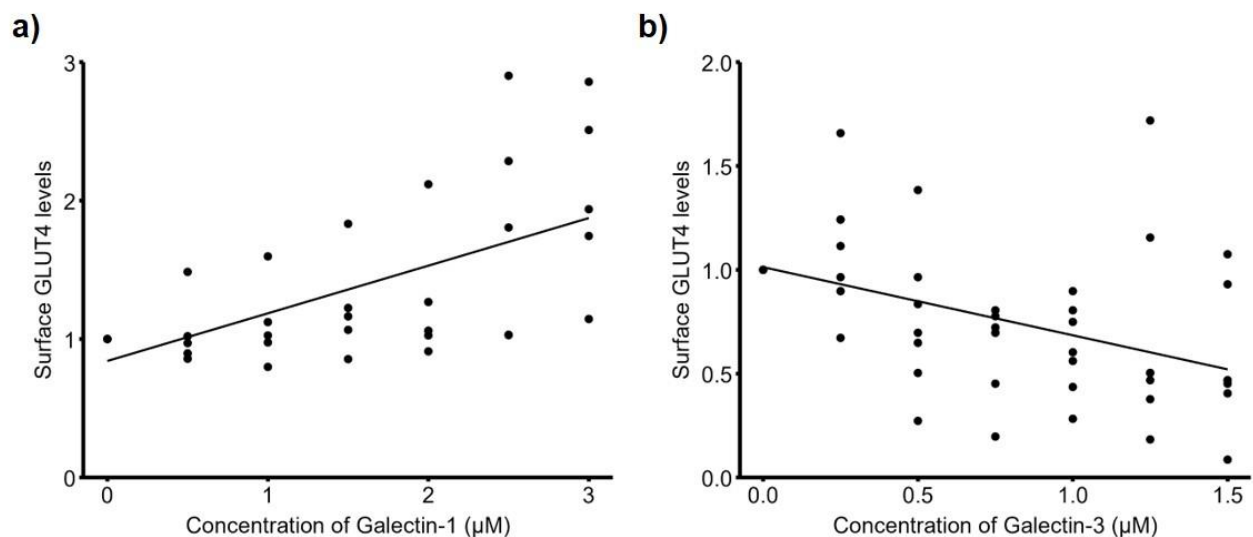


Figure 5-5 – Increasing galectin concentrations significantly changed surface GLUT4 levels

HA-GLUT4-GFP cells were serum starved with serum-free DMEM for 2 hours, in the presence of increasing concentrations of **a)** recombinant galectin-1 (0 μM, 0.5 μM, 1.0 μM, 1.5 μM, 2.0 μM, 2.5 μM, 3.0 μM) or **b)** recombinant galectin-3 (0 μM, 0.25 μM, 0.5 μM, 0.75 μM, 1.0 μM, 1.25 μM, 1.5 μM). Cells were fixed with 2% PFA, and then surface stained with anti-HA.11 antibody (1:1000), and anti-mouse AlexaFluor 647 (1:500). Cells were then collected for flow cytometry. HA:GFP ratio is normalised to 0 μM. **a)** $n=5$, $p<0.01$ **b)** $n=6$, $p<0.01$

5.1.4 Reduction in galectin-3 expression causes significant decrease in surface GLUT4

The mode of action of galectins can be sensitive to changes in concentration (Mathew and Donaldson, 2018). Consequently, it was important to also examine the effects of reducing expression of galectin-1 and -3 to build an accurate picture of galectin action. This was achieved through performing siRNA knockdown of galectin-1 and galectin-3, resulting in a significant reduction of galectin levels (**Figure 5-6a&c**).

Surface GLUT4 levels following siRNA knockdown were measured by flow cytometry, but no significant difference was observed for galectin-1 or -3 knockdown compared to control siRNA (**Figure 5-6b&d**). This could suggest that neither galectin plays an important role in normal conditions. However, siRNA knockdown has limitations, as it does not achieve complete knockout and is transient (up to 72 hours).

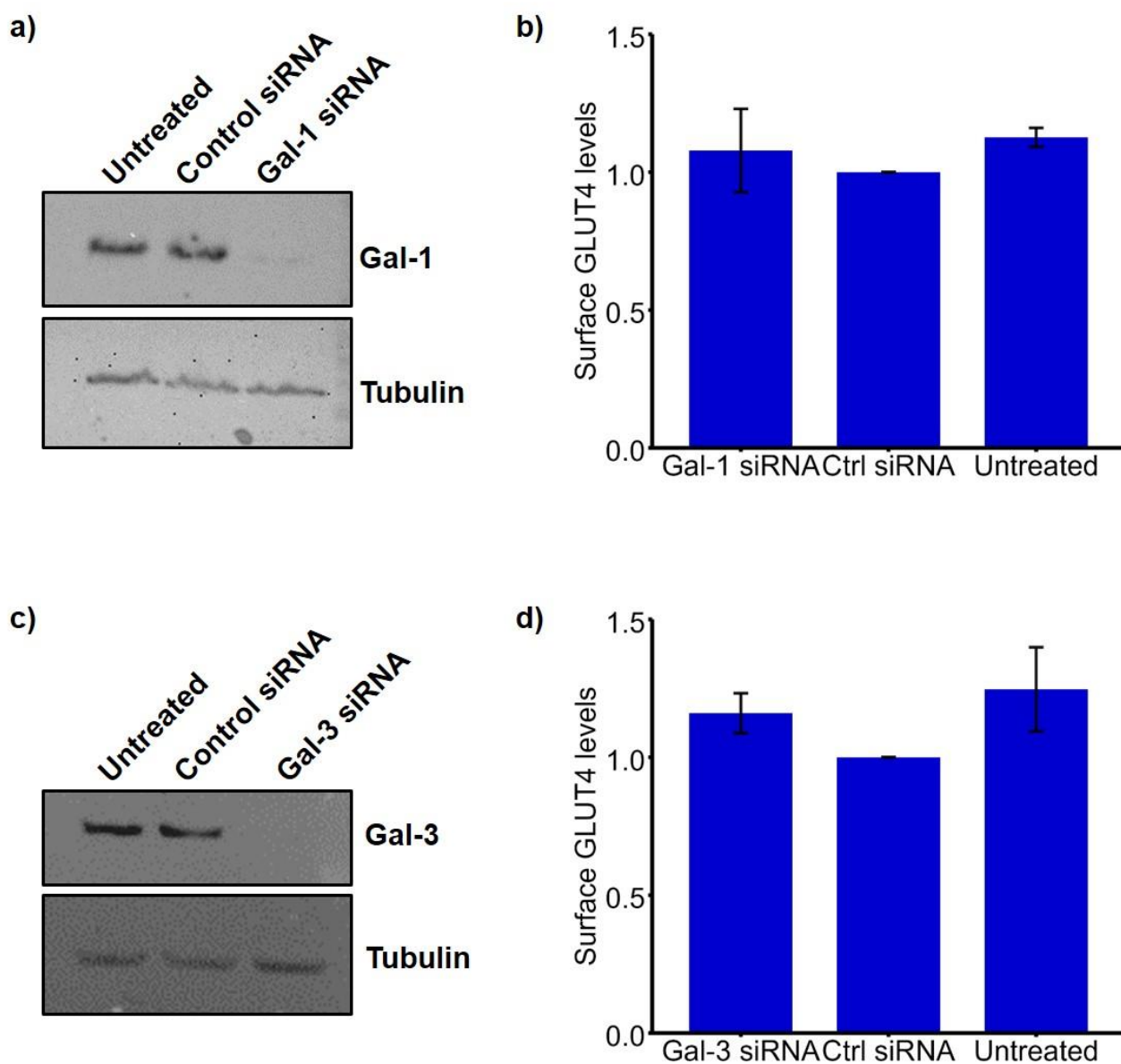


Figure 5-6- Galectin siRNA knockdown effect on surface GLUT4

a&c) HA-GLUT4-GFP expressing Hela cells were transfected with galectin-1 (**a**) or galectin-3 (**c**) siRNA and non-targeting siRNA, or left untreated. Blots shows efficient knockdown for each galectin. **b&d)** siRNA transfected cells were serum starved, then fixed with 2% PFA, and then surface stained with anti-HA.11 antibody (1:1000), and anti-mouse AlexaFluor 647 (1:500). Flow cytometry was then performed to determine ratio of HA:GFP signal. Data normalised to control siRNA, n=4

To circumvent the limitations associated with the siRNA approach, knockout of galectin-3 using the CRISPR-Cas9 system was performed to examine whether a longer term reduction of galectin-3 may affect surface GLUT4. Due to time constraints, only galectin-3 knockout was achieved. Galectin-3 knockout was prioritised based on the well characterised roles it plays both in mediating glycoprotein endocytosis at the cell surface and promoting surface retention (Mathew and Donaldson, 2018). Although galectin-1 has been shown to be capable of crosslinking with glycoproteins at the cell surface, this is not a well characterised role (Belardi et al., 2012).

Two separate CRISPR sgRNA guides were designed and ligated into a px462 Cas9n vector which was transformed into DH5 α *E.coli* cells. A colony PCR was then carried out in order to test the success of the ligation. As shown in **Figure 5-7a**, strong bands were seen for colony 4 & 5 for both sgRNAs, demonstrating successful ligation of the sgRNAs into the vector. DNA purification was then carried out using DNA miniprep, and the DNA sequence was validated through Sanger sequencing (Eurofins).

HA-GLUT4-GFP HeLa cells were transfected with the sgRNA-ligated Cas9n plasmid, and selected using puromycin. Single cell colonies were grown and tested for galectin-3 expression, but complete knockout wasn't achieved in any colonies. However, a colony was identified which had around 50% reduced expression (**Figure 5-7b**). This could be due to the presence of multiple chromosomes (the numbers of which are not known in HeLa due a high degree of genetic variation) (Liu et al., 2019). The 50% reduction in expression suggests that knockout was successful in 50% of the chromosomes. Despite the fact this was only a partial knockout, this still represented a long term reduction of galectin-3, so this cell line was taken forward for further analysis.

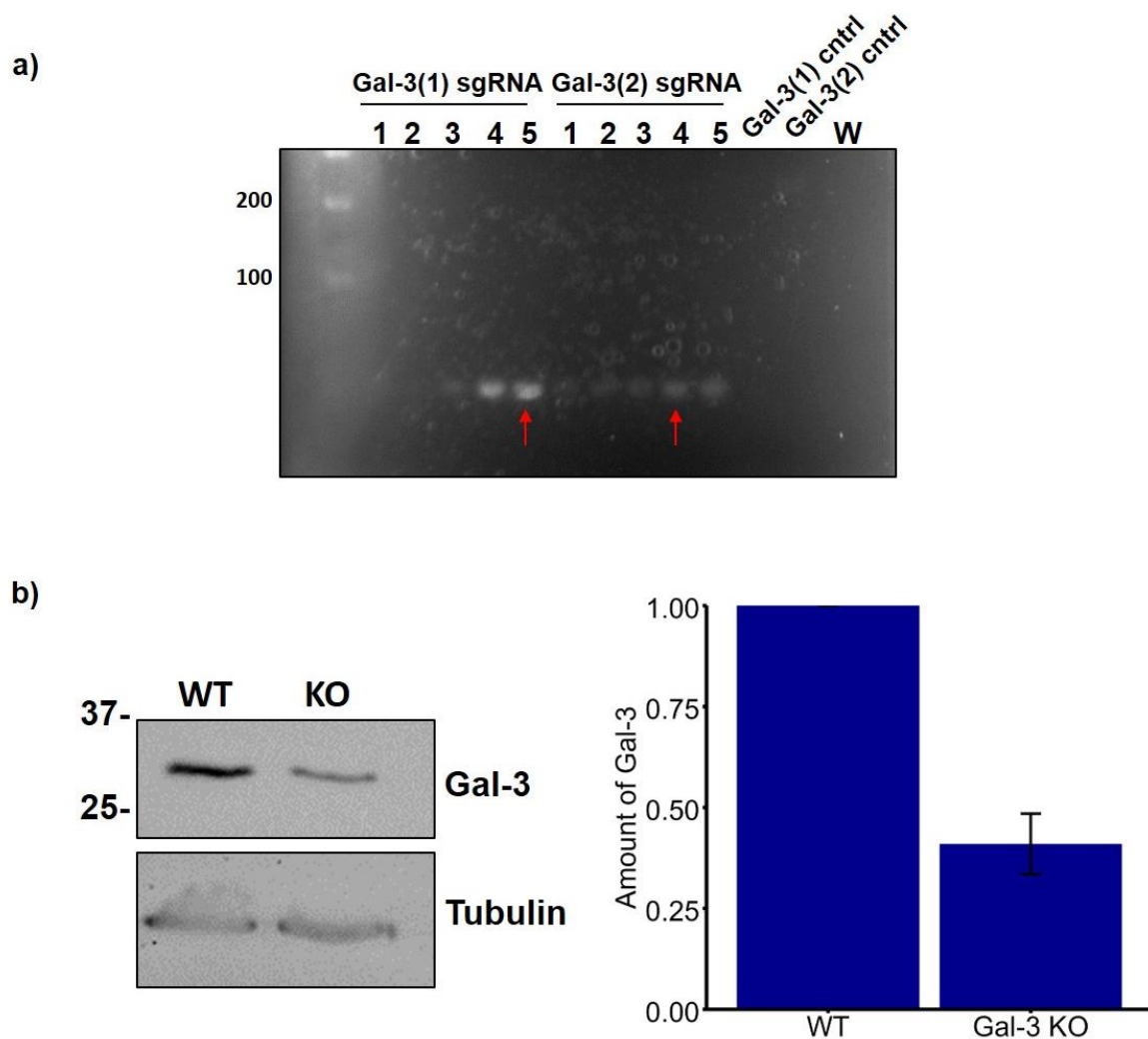


Figure 5-7 - Galectin-3 CRISPR KO

a) Gal-3 sgRNAs were ligated into Cas9n px462 and transformed into DH5α *E.coli* cells. Colony PCR was performed on transformed *E.coli* cells and samples were subjected to DNA electrophoresis with a 3% agarose gel. Red arrows indicate colonies which were taken forward **b)** Following transfection of galectin-3 CRISPR Cas9 plasmids and selection by puromycin, cell colonies were tested for galectin-3 expression by subjecting to western blot analysis with anti-galectin-3 (1:1000) and tubulin (1:1000). Bands were quantified using image J, $n=7$, $p<0.001$

Contrary to expectations, a significant decrease in surface GLUT4 levels was observed in the galectin-3 partial knockout compared to WT (**Figure 5-8**). This was surprising given that increasing galectin-3 concentration also resulted in decreased surface GLUT4 (**Figure 5-5**). However, long term reduction of galectin-3 may be causing compensatory mechanisms to act, through increased expression of other galectins. For example, effects observed through inhibiting galectin-3 have shown to be reversed when inhibition is longer than 20 minutes (personal communication from Ludger Johannes). To avoid this, shorter term inhibition of galectin-3 needs to be performed to establish whether compensatory mechanisms from other galectins cause this reduction in surface GLUT4.

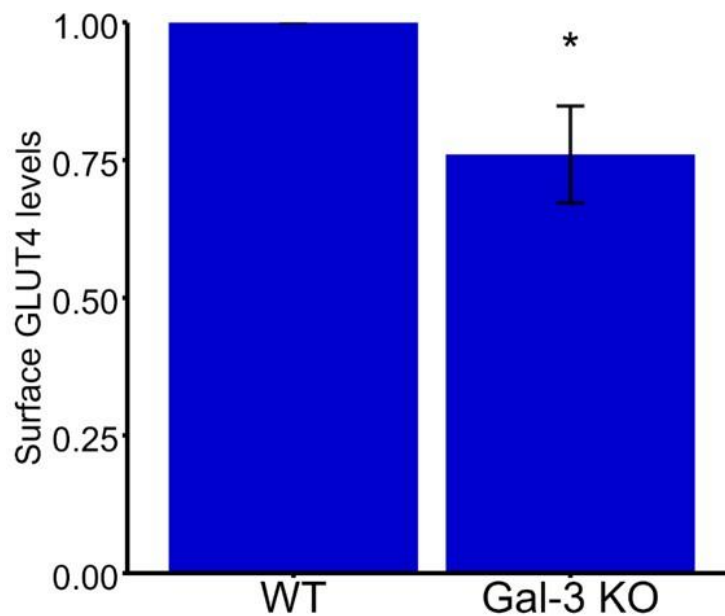


Figure 5-8 - Surface GLUT4 with partial Galectin-3 KO

HA-GLUT4-GFP HeLa cells WT vs Gal-3 KO were serum starved for 2 hours, then fixed with 2% PFA and surface stained with α -HA antibody (1:1000) and α -mouse Alexa 647 (1:500). HA:GFP ratio was measured by flow cytometry to show the proportion of GLUT4 present at the surface. $n = 6$, $p < 0.5$

5.1.5 Transient inhibition of galectin-3 results in significant decrease in surface GLUT4

Small molecule inhibitors of galectin-1 and -3 binding were used in the specific and rapid inhibition of each galectin (Ivashenka et al., 2021; Zetterberg et al., 2018). This can be achieved through treating the cells for 15 minutes, a time-frame which is short enough to avoid compensatory mechanisms through increased protein expression of other galectins. Measuring surface GLUT4 levels following treatment with these inhibitors separately or combined showed that inhibiting galectin-3 binding caused a significant decrease in surface GLUT4 (**Figure 5-9**). This is consistent with decreased surface GLUT4 observed in the partial galectin-3 knockout cells, suggesting that compensation was not a factor in that result (**Figure 5-8**). This suggests that galectin-3 contributes to the cell surface retention of GLUT4, in contrast to previous data indicating that galectin-3 mediates the *N*-glycan's role in intracellular sequestration (**Figure 5-5b**).

Inhibition of galectin-1 binding did not cause a significant decrease in surface GLUT4, and there is no cumulative effect when combining both inhibitors (**Figure 5-9**). This suggests that, despite its ability to cause an increase in surface GLUT4, galectin-1 does not have a specific interaction with GLUT4 in normal conditions.

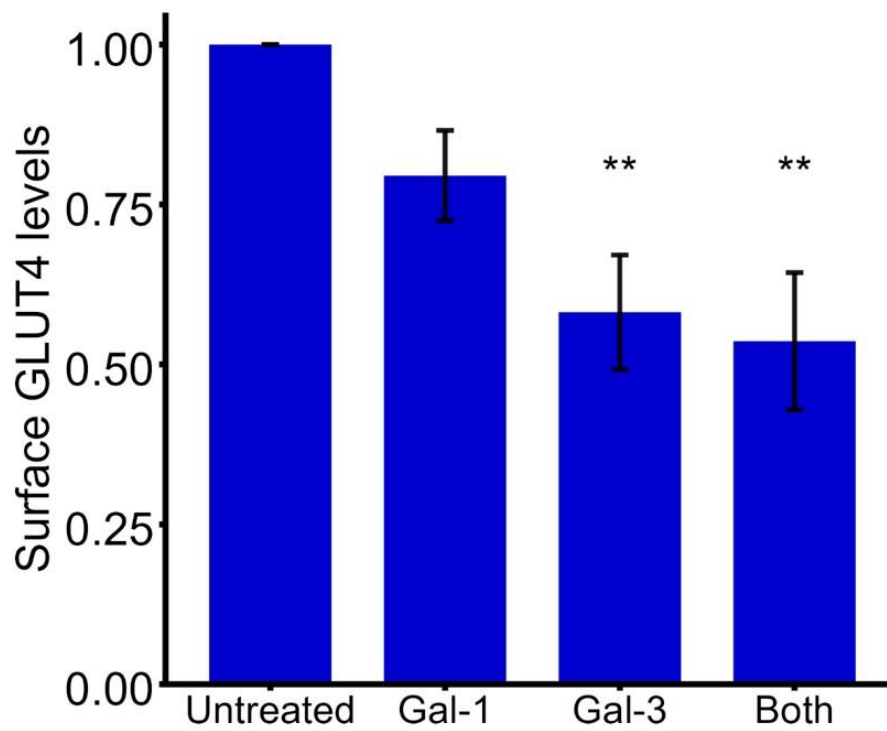


Figure 5-9 - Surface GLUT4 with galectin inhibitors

HA-GLUT4-GFP HeLa cells were treated with 10 μ M galectin inhibitor (or DMSO for untreated) for 15 minutes, following 2 hour serum starvation, and then fixed in 2% PFA and surface stained with α -HA antibody (1:1000) and α -mouse Alexa 647 (1:500). HA:GFP ratio was measured by flow cytometry. $n=4$, ** = $p<0.01$

5.2 Discussion

The finding that *N*-glycosylation plays a role in the intracellular sequestration of GLUT4 combined with the identification of branched poly lactosamine (chapters 3 and 4) led to the hypothesis that the poly lactosamine-binding galectin family binds GLUT4's *N*-glycan to drive its endocytosis. The data collected in this chapter found evidence that both supported this hypothesis and opposed it, suggesting that GLUT4's *N*-glycan functions in more processes than previously predicted.

Addition of lactose has previously been shown to inhibit galectin-glycoprotein interactions (Lakshminarayan et al., 2014). Using the flow cytometry assay described in section 3.1.2, a significant increase in surface GLUT4 levels was observed following addition of excess lactose. This was not observed for the sucrose negative control, and no cumulative effect was observed with lactose and kifunensine, indicating that lactose is acting specifically through GLUT4's *N*-glycan.

Galectin-1 and -3 were subsequently identified as lactose-binding proteins present in HeLa cells. Both of these galectins have previously been implicated in Type-2 diabetes, a pathophysiological state in which GLUT4 traffic is dysregulated (Fryk et al., 2016; Yilmaz et al., 2015). Galectin-1 and -3 therefore became the main candidates of interest for GLUT4 binding.

Increasing concentrations of recombinant galectin-1 caused an increase in surface GLUT4. This indicates that the increased surface GLUT4 observed when galectin interactions are inhibited with lactose is not mediated through galectin-1. No change in surface GLUT4 is observed for specific inhibition of galectin-1, either through siRNA knockdown or a small

molecule inhibitor, supporting the interpretation that it does not play a role in normal conditions in HeLa cells. The GLUT4 surface retention observed with galectin-1 addition is likely due to the artificially high concentrations having a non-specific effect, possibly through outcompeting a more specific interactor.

Addition of recombinant galectin-3 caused a significant decrease in surface GLUT4.

Combining this with the lactose-driven increase of surface GLUT4 initially supported the hypothesis that galectin-3 mediates the endocytosis of GLUT4's *N*-glycan. However, both partial knockout of galectin-3 and treatment with a small molecule inhibitor caused a significant decrease in surface GLUT4, suggesting that it instead acts to retain GLUT4 at the cell surface. One explanation for these contrasting data is that recombinant galectin-3 is non-functional, as no control experiments were performed to confirm that it is able to oligomerise and function as normal. If this is the case, it may be acting as a competitive inhibitor for endogenous galectin-3, explaining the similar results observed in the addition and inhibition of galectin-3.

Although galectin-1 has a much greater abundance in HeLa cells, it is only galectin-3 inhibition which has a significant effect on surface GLUT4, suggesting a specific interaction of galectin-3 with GLUT4's *N*-glycan. However, the data suggests that it does not mediate the role of GLUT4's *N*-glycan in intracellular sequestration (identified in sections 3.1.2 and 3.1.3). This may instead be facilitated by a different galectin or a different protein altogether.

While this project has focused on the basal trafficking of GLUT4, it is possible that a function for galectin-3 in cell surface retention is more relevant in insulin-stimulated cells. It has previously been reported that different mechanisms drive endocytosis in basal and insulin

stimulated conditions (Blot and McGraw, 2006). The *N*-glycan may have different interactors in these different conditions, and thus contribute to both the intracellular sequestration of GLUT4 in basal conditions, and GLUT4 cell surface retention in insulin stimulation. This would be consistent with data showing that while insulin-stimulated GLUT4 translocation is still intact in kifunensine treated cells, GLUT4 residence time on the cell surface is dramatically decreased (Hirayama et al., 2016). Studying the effects on galectin-3 inhibition under insulin stimulation would provide key evidence for this hypothesis.

The mechanisms behind the role of the *N*-glycan in keeping GLUT4 away from the cell surface still remain unclear. Chapter 4 demonstrated the presence of an unusual sugar on GLUT4's *N*-glycan. Given the rare (if not unique) nature of this sugar, it seems likely that there is a functional reason for its presence. Once this sugar has been definitively identified, alternative binding partners may be found which could mediate the endocytosis (or prevent the exocytosis) of GLUT4 in basal conditions.

6 Discussion

6.1 Discussion of results and model for GLUT4's *N*-glycan function

Despite the fact that *N*-glycosylation plays a key role in the regulation of many glycoproteins, GLUT4's *N*-glycosylation has been overlooked compared to the vast amount of research on other aspects of GLUT4 trafficking. Studies which have investigated GLUT4's *N*-glycan used different cell models and had conflicting conclusions, which make the role of GLUT4's *N*-glycan difficult to interpret (Haga et al., 2011; Hirayama et al., 2016; Zaarour et al., 2012). The aim of this project was to elucidate the structure and function of GLUT4's *N*-glycan using a range of methods with one consistent experimental model for GLUT4 trafficking.

The HA-GLUT4-GFP HeLa cell line was chosen as a human cell model which demonstrates insulin-responsive trafficking that is comparable to human adipocytes (Camus et al., 2020; Morris et al., 2020). In addition, using HeLa cells allow for a wide range of techniques to be used due the ease of cell culture and compliance for genetic manipulation.

Using this cell model, three different roles for GLUT4's *N*-glycan were identified, in which its structural features are critical to its function. These are summarised in **Figure 6-1** and in the following sections.

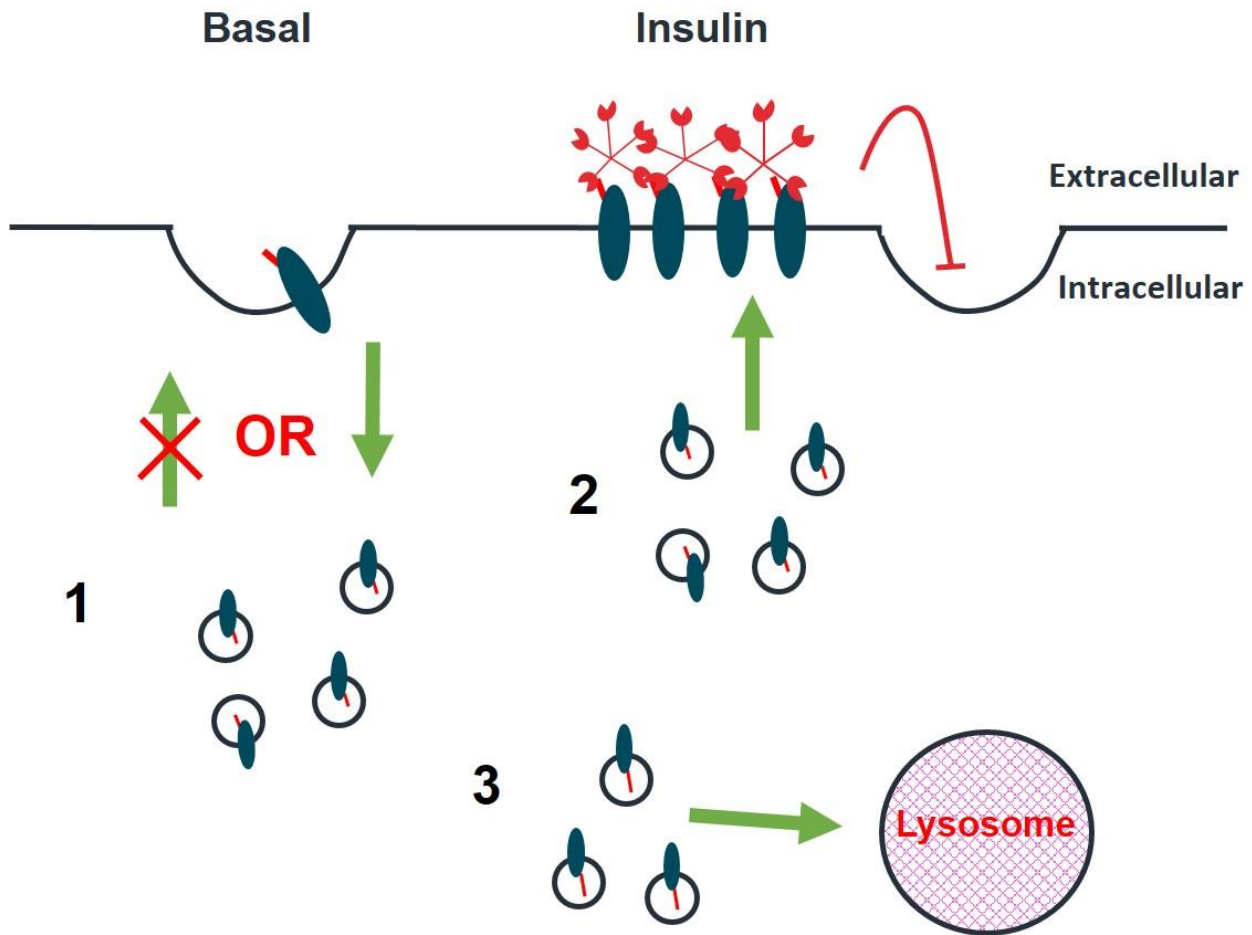


Figure 6-1 - 3 roles for GLUT4's N-glycan

1) GLUT4's N-glycan contributes to its intracellular sequestration either through promoting endocytosis or reducing exocytosis **2)** Galectin-3 mediates the cell surface retention of GLUT4 through binding polylactosamine present in its N-glycan **3)** Glycan size acts as a signal for GLUT4 age. When the N-glycan exceeds a certain size, GLUT4 becomes targeted for degradation

6.1.1 GLUT4's *N*-glycan contributes to the intracellular sequestration of GLUT4 and its cell surface retention

The range of methods used for studying GLUT4's *N*-glycan produced results which sometimes appeared conflicting, as summarised in **Table 6-1**. These results can be reconciled by a model whereby the *N*-glycan plays two opposite roles in basal vs insulin conditions.

Table 6-1 – Summary of effects for altering GLUT4's *N*-glycan structure or inhibiting galectin

Treatment	Effect on surface GLUT4	Role for <i>N</i> -glycan/galectin it suggests
Kifunensine or swainsonine	↑	Intracellular sequestration
Lactose and maltose	↑	Intracellular sequestration
Increasing galectin-1 conc.	↑	Cell surface retention
Increasing galectin-3 conc.	↓	Intracellular sequestration
Galectin-1 siRNA knockdown	—	—
Galectin-3 siRNA knockdown	—	—
Partial Galectin-3 knockout	↓	Cell surface retention
Galectin-1 small molecule inhibitor	—	—
Galectin-3 small molecule inhibitor	↓	Cell surface retention

Flow cytometry and subcellular fractionation data showed an increase in surface GLUT4 when the *N*-glycan structure was altered, which demonstrated that GLUT4's *N*-glycan contributes to the intracellular sequestration of GLUT4 (**sections 3.1.2 and 3.1.3**). While the focus of previous studies was on GLUT4's insulin response, they also observed a surface GLUT4 increase in basal conditions when the *N*-glycan was removed/altered (Haga et al., 2011; Zaarour et al., 2012). Data presented in **section 3.1.2** also agreed with previous studies in respect to the loss of insulin response with altered *N*-glycan structure, suggesting that the *N*-glycan is important in both conditions (Haga et al., 2011; Ing et al., 1996). However, the focus of this project was mainly on the effect observed at basal conditions.

Previous studies on GLUT4's *N*-glycan have either mutated the Asn57 glycosylation site or inhibited mannosidase I with kifunensine. To our knowledge, this is the first study to use the mannosidase II inhibitor swainsonine in the study of GLUT4's *N*-glycan. Although no change in GLUT4 molecular weight was observed, swainsonine treatment caused an increase in surface GLUT4 levels. This indicates that swainsonine alters *N*-glycan structure without reducing its size, and that this specific structure is key to its function. It also indicates that GLUT4's *N*-glycan is in the complex form, as mannosidase II acts to facilitate the transition of an *N*-glycan from a hybrid form to a complex form.

The effects observed with swainsonine underscored the importance of determining the structure of the *N*-glycan. Following optimisation of both GLUT4 purification and reverse-phase HPLC linked to fluorescence detection, monosaccharide composition analysis of GLUT4 was performed. Coupled with the finding that the *N*-glycan is in the complex form, monosaccharide peaks were quantified to reveal an approximate structure of $\text{Man}_3\text{GlcNAc}_{12}\text{Gal}_{10}\text{Sia}_7\text{Fuc}$. The amount of galactose and GlcNAc present compared to

mannose confirmed the presence of polylactosamine. Sialic acid addition tends to occur on terminal galactose monomers. To generate enough terminal ends for the number of sialic acid residues present, polylactosamine must be branched.

The β -galactoside binding galectin family display increased affinity for branched polylactosamine compared to linear polylactosamine or single N-acetyllactosamine units (Hirabayashi et al., 2002). The discovery of branched polylactosamine on GLUT4's N-glycan supported the hypothesis that a member of the galectin family binds to GLUT4 and influences its trafficking. This was supported by the increase in surface GLUT4 observed in the presence of excess lactose, which has been shown to inhibit galectin binding (Lakshminarayan et al., 2014). Galectin-1 and -3 were identified as lactose-binding proteins present in HeLa cells, which had previously been identified as proteins of interest based on their association with type 2 diabetes (Fryk et al., 2016; Weigert et al., 2010; Yilmaz et al., 2015). In addition, roles for galectin-3 both in facilitating clathrin-independent endocytosis and in cell surface retention of glycoproteins have previously been reported, along with a less well characterised role for galectin-1 in cell surface retention (Belardi et al., 2012; Mathew and Donaldson, 2018).

As summarised in **Table 6-1**, the effects observed when investigating galectin-1 and -3 sometimes appeared conflicting. This may be due to the sensitivities of galectins' mode of action to changes in concentration (Mathew and Donaldson, 2018). For this reason, the results need to be interpreted in the context of all the effects observed.

Adding increasing concentrations of recombinant galectin-1 and -3 to the cell had opposite responses – causing an increase and decrease in surface GLUT4 respectively. Time constraints prevented a CRISPR-Cas9 mediated knockout for galectin-1 being achieved.

However, neither siRNA knockdown nor a specific galectin-1 inhibitor caused a change in surface GLUT4, suggesting that it does not play an important role in regulating surface expression of GLUT4 in HeLa cells. The effects seen through increased concentration galectin-1 is non-physiological in HeLa cells, and it seems that the physiological concentration is not sufficient to have a functional effect.

Combining the observation that recombinant galectin-3 drives the internalisation of surface GLUT4 with the finding that excess lactose increases surface GLUT4 initially suggested that galectin-3 promotes GLUT4 internalisation through its previously identified role in the generation of clathrin-independent carriers (Lakshminarayan et al., 2014). However, specifically inhibiting galectin-3 through partial knockout or using a small molecule inhibitor also caused a decrease in surface GLUT4. The similarity in results observed with recombinant galectin-3 addition and inhibition of galectin-3 suggested that, rather than supplementing endogenous galectin-3 action, recombinant galectin-3 acted as a competitive inhibitor. Galectin-3's ability to regulate surface glycoproteins is dependent on its oligomerisation, but the ability of recombinant galectin-3 to oligomerise and function as normal was not tested. If it is acting as a competitive inhibitor, the data is consistent with a role for galectin-3 in the cell surface retention of GLUT4.

When sugar solutions were added to HA-GLUT4-GFP HeLa cells, only the disaccharides lactose and maltose had an effect on surface GLUT4, both causing a significant increase. Maltose is not a β -galactoside sugar, so was not expected to inhibit galectin interactions and was originally included as a negative control. Its effect on surface GLUT4 could indicate that maltose inhibits a non-galectin protein that binds to GLUT4's *N*-glycan, which can also be inhibited by lactose. If this protein exists, the effects observed on surface GLUT4 suggest

that it would act to promote the intracellular sequestration of GLUT4, rather than the cell surface retention function observed for galectin-3.

A key characteristic of GLUT4 trafficking is the insulin-stimulated redistribution of GLUT4 to the cell surface from an intracellular pool (Bryant et al., 2002). The mechanisms controlling its endocytosis and intracellular trafficking differ in basal vs insulin stimulated conditions. A key example of this is the switch from clathrin-independent to clathrin mediated endocytosis in basal and insulin conditions respectively (Blot and McGraw, 2006). Based on the data, GLUT4's *N*-glycan may contribute to opposite functions in these different conditions – one that facilitates the intracellular sequestration of GLUT4 in basal conditions, and one that facilitates the cell surface retention of GLUT4 with insulin-stimulation (**Figure 6-1**).

While the effects of galectin-3 on the cell surface retention of GLUT4 were only studied in basal conditions, this may become more important in insulin-stimulated cells, when the amount of GLUT4 at the cell surface is vastly increased (Bryant et al., 2002). This would be consistent with data demonstrating that kifunensine treated cells still possess insulin-stimulated GLUT4 trafficking to the cell surface, but do not exhibit cell surface retention (Hirayama et al., 2016). This suggests that the loss of insulin response observed in kifunensine treated cells in section 3.1.2 and previous studies is due to the loss of retention rather than a loss of exocytosis (Haga et al., 2011; Ing et al., 1996). Data looking at the inhibition of galectin-3 in insulin stimulated cells would be key to providing evidence for such a model in GLUT4.

Although GLUT2 is not subject to insulin-stimulated trafficking, there has been evidence that GLUT2's *N*-glycan (also present on the first extracellular loop) also functions in cell surface

retention, possibly mediated by galectin-9 (Ohtsubo et al., 2005). In addition, GLUT1's *N*-glycan also has a polylactosamine chain, indicating it could also be interacting with a galectin (Endo et al., 1990). This could suggest that the *N*-glycan's function in cell surface retention is common across the GLUT family.

The role of GLUT4's *N*-glycan in promoting intracellular sequestration is only observed in basal conditions, and there is currently no evidence for whether this is mediated through promoting endocytosis or preventing exocytosis. GLUT4's intracellular sequestration in basal conditions is an attribute not common across the GLUT family (Bryant et al., 2002). This suggests that the *N*-glycan's role in keeping GLUT4 away from the cell surface is mediated through a unique attribute of its structure. Along with the discovery of branched polylactosamine, structural analysis revealed the presence of a monosaccharide not previously found in mammalian cells. More work is needed to identify this monosaccharide, although there is evidence pointing towards an amino tetrose sugar. Erythrosamine represents the best candidate for this, as although it hasn't been found in mammalian cells, it has previously been found in biology (Fujii and Maruoka, 1982). This could represent a structural aspect that is key to the role of GLUT4's *N*-glycan in intracellular sequestration, and requires further research to combine these structural and functional features.

6.1.2 GLUT4's *N*-glycan size acts a signal for GLUT4 age

In addition to the two roles already described, chapter 3 identified a potential third process in which GLUT4's *N*-glycan plays a key role (**Figure 6-1**). Higher steady state levels of GLUT4 were observed with kifunensine treated cells compared to untreated, but not in swainsonine treated cells. This suggested that GLUT4's *N*-glycan was involved in targeting GLUT4 for degradation, in a role that was dependent on its size rather than the specific structure. A hypothesis was formed that the size of the *N*-glycan acts as a measure of age and once it exceeds a certain size, GLUT4 is directed to the degradation pathway.

GLUT4 has previously been reported to travel through the TGN in its recycling loop (Martin et al., 2000a; Shewan et al., 2003; Slot et al., 1997). A ubiquitin resistant GLUT4(7K/R) mutant which is retained in the TGN (Lamb et al., 2010) had a decrease in molecular weight compared to WT GLUT4. This suggests that GLUT4's *N*-glycan undergoes further processing each time it travels through the TGN. Further evidence for this is the previous finding that IRAP becomes re-sialylated following sialic acid removal with the enzyme neuraminidase and re-entry into the TGN (Shewan et al., 2003). IRAP follows the same trafficking itinerary as GLUT4, suggesting that GLUT4's transport through the TGN could involve *N*-glycan processing (Kandror et al., 1994). This supports a model whereby GLUT4's *N*-glycan undergoes sequential glycan processing over time, until it reaches a size which targets it for degradation.

In conclusion, the work to understand the function of GLUT4's *N*-glycan has revealed roles in 3 different processes. The first is the intracellular sequestration of GLUT4 in basal conditions, the second in the cell surface retention of GLUT4 under insulin stimulation, and the third in the eventual targeting of GLUT4 for degradation. Furthermore, structural

analysis has revealed that GLUT4's *N*-glycan has a complex structure with a branched polylactosamine. It also contains a monosaccharide species present not previously found in mammalian cells, which may have an amino tetrose structure.

6.2 Future work

Further work is required to better characterise the role of GLUT4's *N*-glycan in each of the three functions identified, and to further elucidate the structure (**Figure 6-1**).

There is currently no evidence for whether the role of GLUT4's *N*-glycan in intracellular sequestration is due to inhibiting exocytosis or promoting endocytosis. The ability to track GLUT4 over time may help answer this. For example, fluorogenic probes have been previously used to track GLUT4 movement to the cell surface, visualising GLUT4 which has been present at the cell surface through the use of cell-impermeable probes (Hirayama et al., 2016). A similar experiment could be used to observe whether untreated GLUT4 has decreased labelling compared to kifunensine treated GLUT4, which would indicate that the *N*-glycan acts to prevent exocytosis. Conversely, if no difference is seen, then this may indicate that the *N*-glycan is acting to promote endocytosis. This could be performed using the RUSH system to track one pool of GLUT4 over time.

It was shown that the structure of GLUT4's *N*-glycan is crucial to its role in intracellular sequestration. Elucidating the full structure is key to understanding this function, a crucial aspect of which is definitively identifying the unknown monosaccharide. A key candidate for this is erythrosamine. The synthesis of erythrosamine (from 2-amino-2-deoxy-D-erythronic acid) is relatively simple, and would provide a standard to compare against the large peak observed in GLUT4. In addition, information as to the location of this monosaccharide on the *N*-glycan structure could be elucidated by cleaving off the polylactosamine with an endogalactosidase, and performing HPLC on the polylactosamine pool vs the rest of the *N*-glycan.

The degree of branching linkages that connect the monosaccharide residues also require elucidation in order to build an accurate picture of the *N*-glycan structure. For example, the approximate structure for GLUT4's *N*-glycan currently includes two antennae on the α 1,3-branch, which is an assumption rather than backed up by evidence.

With respect to the cell surface retention role mediated by galectin-3, evidence for its importance to GLUT4's insulin response could be determined through inhibiting galectin-3 binding in the presence of insulin stimulation. Inhibition of galectin-3 can be performed in the presence of small molecule inhibitors, as well as through siRNA-mediated knockdown had no effect on surface GLUT4. Insulin usually stimulates a large increase in surface GLUT4, so the importance of galectin-3 to this function can be assessed from the extent to which this is reduced. This may also reveal differences in surface GLUT4 not previously observed with the siRNA knockdown.

Completing the GLUT4 RUSH assay would provide key evidence for whether its *N*-glycosylation occurs in a sequential manner, as it allows the tracking of one homogenous pool of GLUT4 over time. This is crucial to supporting the hypothesis that GLUT4's *N*-glycan increases in size over time until it targets GLUT4 for degradation. Some evidence for this has been supplied through the molecular weight shift observed for the ubiquitin-resistant GLUT4(7K/R) mutant. However, this assumes that the molecular weight shift is because of the loss of *N*-glycan. PNGase F treatment can be performed on the sample which cleaves *N*-glycans off their glycoprotein. If the molecular weight does not decrease further, this would show that GLUT4(7K/R)'s *N*-glycan is not the full size observed in WT GLUT4.

In the longer term, the potential of GLUT4's *N*-glycan as a therapeutic target can be explored further. Its role in cell surface retention under insulin stimulation may prove

particularly important in the reduced insulin response observed in people living with type 2 diabetes. Finding artificial methods of prolonging GLUT4's presence on the cell surface may facilitate the glucose uptake needed when blood glucose levels are elevated.

This could also be achieved by finding ways to overcome the intracellular sequestration mechanisms of GLUT4. The current model suggests that the unknown monosaccharide found on GLUT4's *N*-glycan may mediate this function. Although this remains to be identified, evidence suggests that this is a monosaccharide not previously identified in mammalian cells, which would provide a unique target. Identifying this monosaccharide and its interactors could provide a mechanism to inhibit the intracellular sequestration of GLUT4.

Appendices

Appendix I

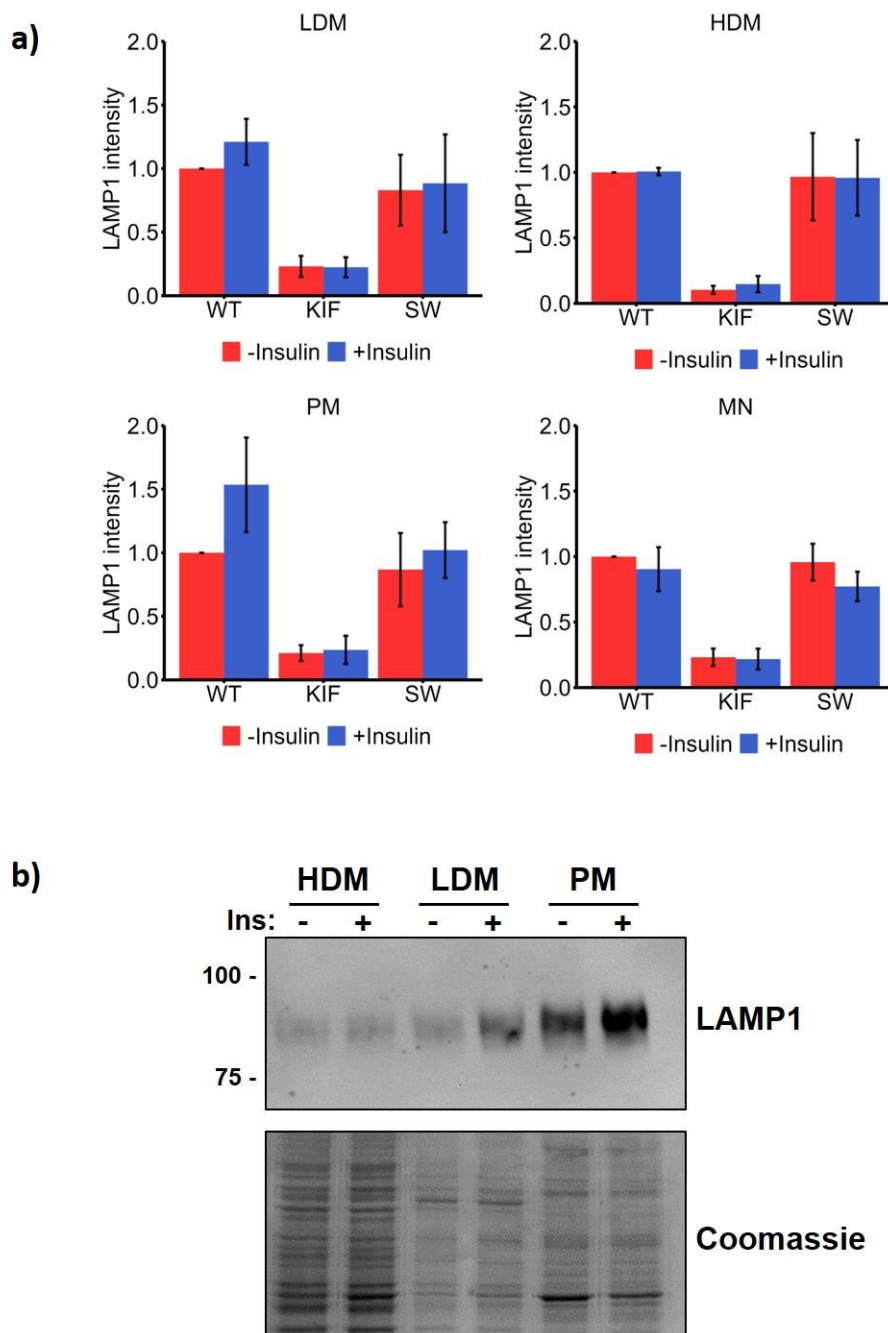


Figure I - LAMP1 is insulin-responsive and its glycosylation prevents its degradation
a) LAMP1 band intensities were measured using ImageJ across 5 separate experiments. LDM = low density microsomes, HDM = high density microsomes, PM = plasma membrane, MN = mitochondrial and nuclear fraction. **b)** Subcellular fractionation of MSC-derived adipocytes (performed by Dimi Kioumourtzoglou) Western blot performed on 10% SDS-PAGE gel and probed against LAMP1 (1:1000). SDS-PAGE gel was coomassie stained to show equal loading.

Appendix II

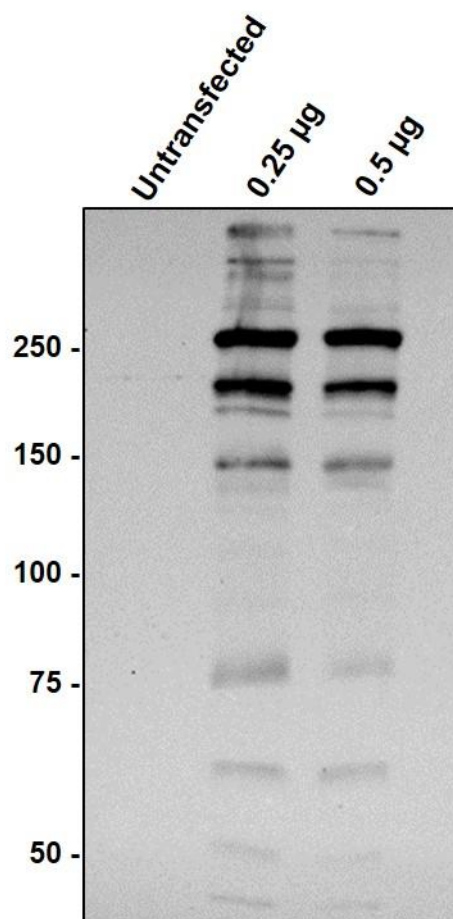


Figure II - Transient transfection of HA-GLUT4-SBP-GFP

HeLa cells were transfected with either 0.25 or 0.5 µg of HA-GLUT4-SBP-GFP RUSH plasmid, or left untransfected. After 72 hours, cells were harvested in 1X sample buffer and subjected to western blot analysis following separation by SDS-PAGE, and were probed using anti-HA.11 (1:1000).

Appendix III

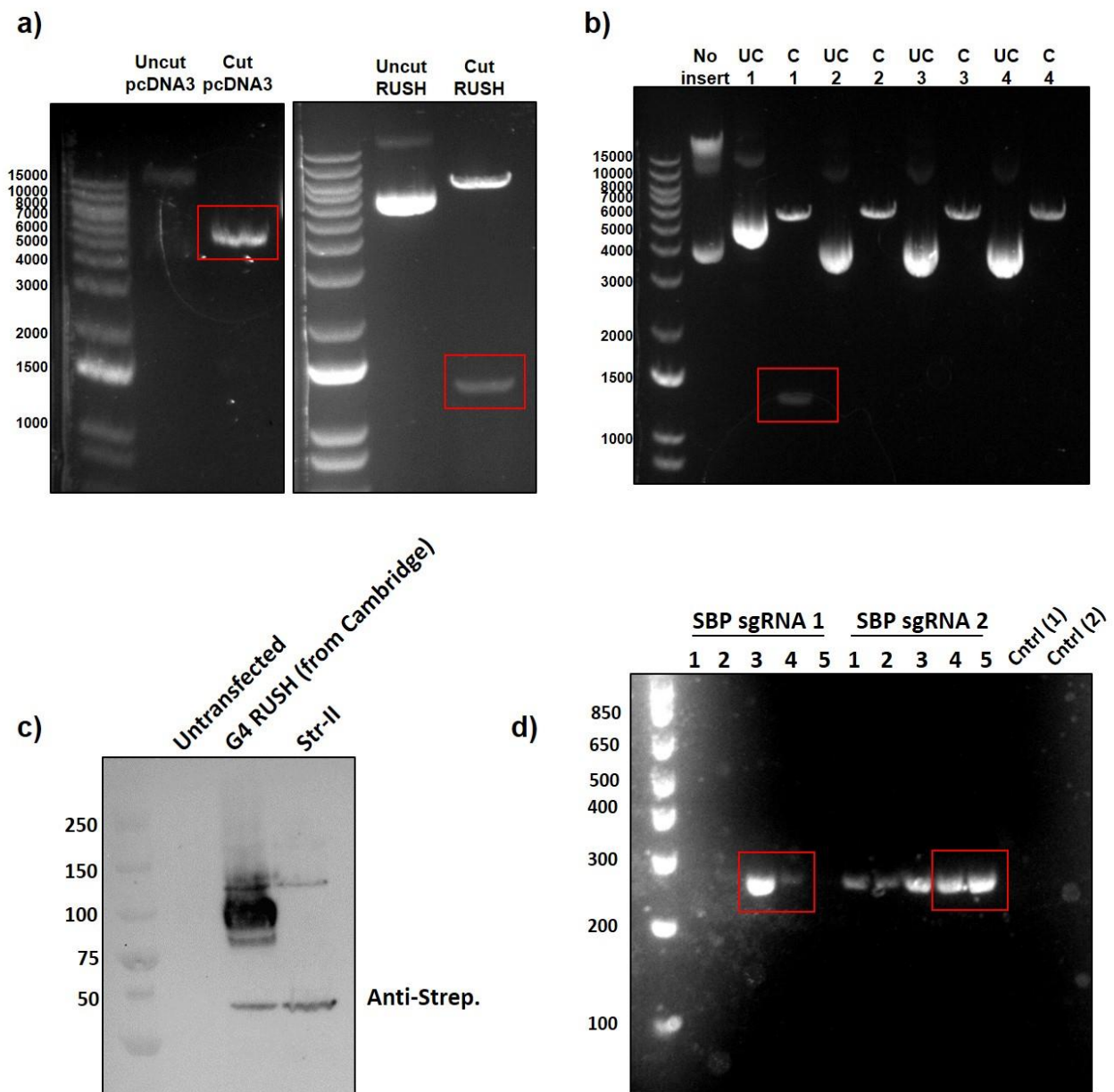


Figure III - Development of a Str-II stable cell line

a) pcDNA3 and HA-GLUT4-SBP_Str-II RUSH plasmid were restriction digested with EcoRV-HF and NotI-HF, and 1% agarose DNA gel was run to confirm digestion was successful **b)** pcDNA3 vector and Str-II insert were ligated together and transformed into *E.coli*. Colonies were picked, and restriction digest with BamHI-HF was performed to confirm presence of insert. Uncut (UC) and cut (C) vectors were run on 1% agarose gel. Red box indicates presence of insert in colony 1. **c)** HA-GLUT4-GFP HeLa cells were stably transfected with Str-II pcDNA vector and ran on 12% DNA gel alongside untransfected HA-GLUT4-GFP cells, and stable RUSH cells containing Str-II from Cambridge. Blot was probed with anti-streptavidin (1:1000) **d)** SBP sgRNAs were annealed and ligated into a Cas9n px462 vector

Appendix IV

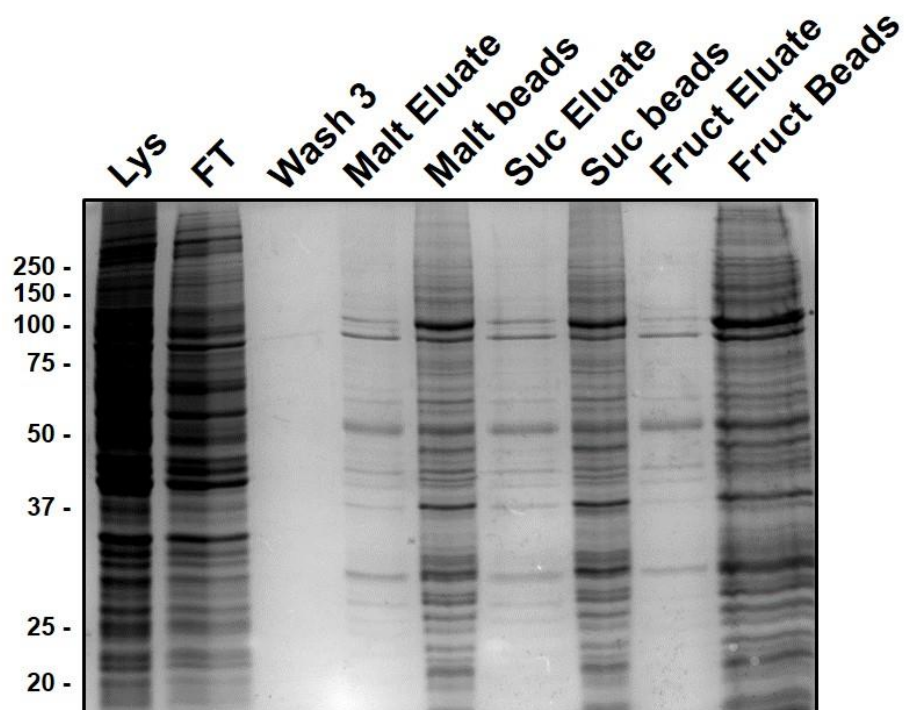


Figure IV - Maltose pulldown assay

HA-GLUT4-GFP Hela cells were incubated with maltose beads and washed to remove unbound proteins. Bound proteins were then eluted with maltose, sucrose or fructose, and run on a 15% SDS-PAGE gel. This was then silver-stained in order to visualise the protein bands

Abbreviations:

AA	Anthranilic acid
ATP	Adenosine 5'-triphosphate
AMP	Ampicillin
APS	Ammonium persulfate
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
CHO	Chinese Hamster Ovary
CIE	Clathrin-independent endocytosis
CLIC	Clathrin independent carrier
CME	Clathrin-mediated endocytosis
CRD	Carbohydrate recognition domain
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol

EEA	Early endosome antigen 1
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FRET	Förster resonance energy transfer
GFP	Green Fluorescent Protein
GL-Lect	GlycoLipids and Lectins
GLUT	Glucose Transporter
GSC	GLUT4 storage compartment
GSLs	Glycosphingolipids
HA	Hemagglutinin
HDM	High density microsomes
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazine] ethanesulfonic acid
HRP	Horseradish peroxidase
HPLC	High-performance liquid chromatography
IRAP	Insulin-responsive aminopeptidase
IRVs	Insulin responsive vesicles

KO	Knock-out
LAMP1	Lysosomal-associated membrane protein 1
LB	Lysogeny Broth
LC-MS	Liquid chromatography–mass spectrometry
LDM	Low density microsomes
LMNG	Lauryl Maltose Neopentyl Glycol
MAN1/2	Mannosidase 1/2
MN	Mitochondrial and Nuclear fraction
MSCs	Mesenchymal stem cells
NHS	N-hydroxysuccinimide
Ni-NTA	Nickel-Nitriloacetic acid
NTP	Nucleoside Triphosphate
OPD	O-Phenylenediamine
OST	Oligosaccharyltransferase
PBS	Phosphate buffered saline
PM	Plasma membrane
PNGase F	Peptide:N-glycosidase F

PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RUSH	Retention Using Selective Hooks
SBP	Streptavidin Binding Protein
SDS	Sodium Dodecyl-Sulfate
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SOC media	Super Optimal broth with Catabolite repression media
ssODN	Single-stranded Oligodeoxynucleotides
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TAE	Tris-acetate-EDTA
TEMED	N,N,N',N'-Tetramethyl ethylenediamine
TFA	Trifluoroacetic acid
TfR	Transferrin Receptor
TGN	Trans Golgi Network
WT	Wild-type

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