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Investigating the Molecular Mechanism of GLUT4 Trafficking

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I. Abstract

The insulin-stimulated glucose transporter GLUT4 is essential to maintaining metabolic homeostasis in mammalian cells. Regulated trafficking of GLUT4 to and from the plasma membrane relies on a complex molecular mechanism integrating insulin signaling and vesicle transport. Understanding this pathway becomes increasingly crucial as Type II Diabetes, a disease characterized by insulin resistance, becomes more prevalent in the population. Despite decades of research, the molecular mechanism of GLUT4 trafficking is not yet fully understood. CRISPR-Cas9 allows for the ablation of individual genes so their role in GLUT4 trafficking can be investigated. A flow cytometry-based assay is used in this study to assess the effects of gene knockout. This project describes the creation of six knockout cell lines to study the role of EXOC3, EXOC7, OSBPL8, OSBPL10, tomosyn-1, and tomosyn-2 in GLUT4 trafficking. Based on the results of these experiments, it appears EXOC7 acts as a positive regulator of GLUT4 translocation while OSBPL8, OSBPL10, tomosyn-1, and tomosyn-2 act as negative regulators.

II. Introduction

The glucose transporter GLUT4 is an essential regulator of metabolic homeostasis. It is a member of the GLUT family of proteins, which transport various sugars across the membrane in a multitude of cell types. The GLUT proteins are comprised of approximately 500 amino acid residues and have 12 membrane-spanning domains (Mueckler et.al. 2013). Fourteen GLUT proteins are expressed in humans, encoded by the SLC2 genes. GLUT4, encoded by SLC2A4, is an insulin-stimulated glucose transporter present primarily in adipocytes and skeletal muscle (Huang et.al. 2007). Under basal (low insulin) conditions, GLUT4 is sequestered in 50 nm intracellular storage vesicles referred to as GLUT4 Storage Vesicles (GSVs) (Hashiramoto et.al. 2000). Some GLUT4 resides on the cell surface at basal conditions, but insulin signaling increases plasma membrane levels of GLUT4 5- to 30-fold (Stockli et.al. 2011).

Insulin-stimulated GLUT4 exocytosis plays a critical role in the tight regulation of blood glucose concentration. When carbohydrates are ingested and blood glucose concentration rises, pancreatic β-cells produce insulin, which is released into the bloodstream and circulated throughout the body. In fat and skeletal muscle, insulin binds its receptor and induces a signaling cascade causing translocation of GLUT4 to the surface of the plasma membrane. Upon the termination of insulin signaling, GLUT4 undergoes endocytosis and is sorted back into intracellular storage vesicles (Bryant et.al. 2002). Although the transport of GLUT4 is critical to maintain nutrient homeostasis, the molecular mechanism behind GLUT4 trafficking remains largely uncharacterized.

Previous research has shown the GLUT4 transport pathway to be incredibly complex, involving intracellular cycling of GLUT4 between the *trans*-Golgi network, the endosomal system, and the plasma membrane (Bryant et.al. 2002). The intersection between insulin

signaling and vesicle transport machinery adds another layer of complexity. Insulin signaling impacts several cellular processes including cell growth and differentiation, protein metabolism, and lipid metabolism (De Meyts 2016). The insulin receptor, a member of the receptor tyrosine kinase family, phosphorylates several proximal substrates upon activation as shown in Figure 1. Most important among these substrates in relation to glucose uptake are members of the insulin-receptor-substrate family, IRS-1 and IRS-2 (Myers et.al. 1996). Tyrosine-phosphorylated IRS proteins recruit the effector molecule phosphoinositide 3-kinase (PI3K), which converts phosphatidylinositol-4,5-diphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), allowing for the recruitment of phosphoinositide-dependent kinase 1 (PDK1) (Whiteman et.al. 2003). At the plasma membrane, PDK1 and mTORC2 phosphorylate AKT. Phosphorylated AKT phosphorylates the GTPase activating protein AS160, which promotes GSV translocation. In parallel, the activated insulin receptor acts through another pathway, phosphorylating c-CBL to

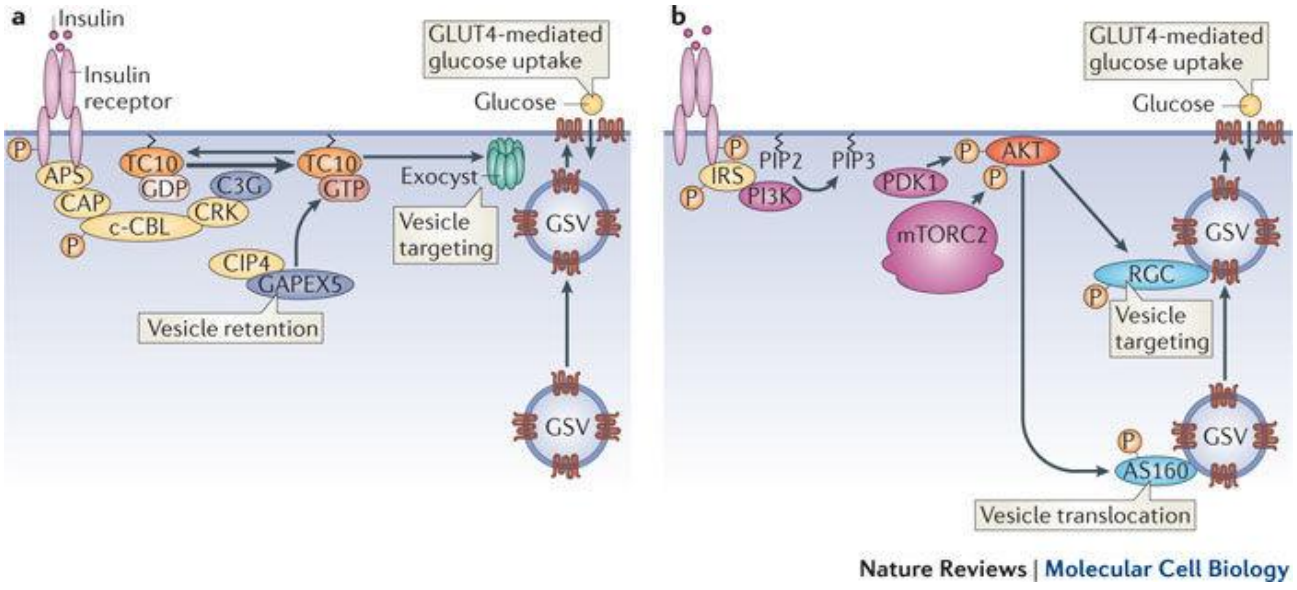

Figure 1: Simplified schematic of insulin signaling through the (a) APS pathway and the (b) PI3K pathway, resulting in GLUT4 translocation (Leto et.al. 2012).

activate TC10, which interacts with the exocyst complex to create target sites for GSVs at the plasma membrane (Leto et.al. 2012).

Once GSVs are translocated, the assembly of SNARE complexes comprised of VAMP2, SNAP23, and syntaxin-4 drives fusion with the plasma membrane (Rea 1998). Fusion is regulated by Munc18C, Doc2b and other SNARE regulators. Upon the termination of insulin signaling, GLUT4 is internalized through endocytosis. Adipocytes sequester GLUT4 through clathrin-mediated endocytosis as well as cholesterol-dependent endocytosis, while muscle cells have mainly been shown to use the clathrin-mediated pathway. In clathrin-mediated endocytosis, the adaptor protein AP2 recruits clathrin to the plasma membrane and interacts with the amino terminus of GLUT4 (Owen et.al. 2004). Cholesterol-dependent endocytosis relies on caveolin, a membrane-deforming protein localized in lipid rafts (Shigematsu et.al. 2003). Vesicles formed by both pathways move towards the cell interior on dynein motors associated with microtubules. After endocytosis, GLUT4 is recycled by endosomes, allowing each GLUT4 molecule to undergo several rounds of translocation (Govers et.al. 2004).

GLUT4 trafficking is immensely complex, involving molecules implicated in both signal transduction and vesicle transport. The pathway is partially described, but more research is needed to determine the mechanistic ways in which these proteins interact. In addition, novel regulators of GLUT4 translocation are discovered frequently, suggesting the pathway is not fully understood. There is far less known about the pathway downstream of AKT, so research into GLUT4 trafficking remains an important area of study.

Understanding GLUT4 translocation at a molecular level becomes ever more crucial as the prevalence of Type II Diabetes skyrockets. Type II Diabetes is characterized by insulin resistance, leading to hyperglycemia and a variety of comorbid conditions (Warram et.al. 1990).

Roughly 30.3 million Americans suffer from diabetes, and Type II Diabetes accounts for approximately 95% of all cases ("National Diabetes Statistics Report" 2017). The underlying cause of this disease is unknown, but diagnosis is strongly correlated to obesity. Approximately 80% of patients with Type II Diabetes are overweight or obese (Daousi et.al. 2006). It has been suggested the link between obesity and insulin resistance may lie in proinflammatory cytokines, disrupted fatty acid metabolism, or endoplasmic reticulum dysfunction (Eckel et.al. 2011). Seeing as insulin signaling and GLUT4 translocation involve such a diverse array of proteins, it is possible obese patients experience disruption at one or more points in the pathway. Elucidating the complete mechanism of GLUT4 trafficking is essential in understanding the link between obesity and insulin resistance.

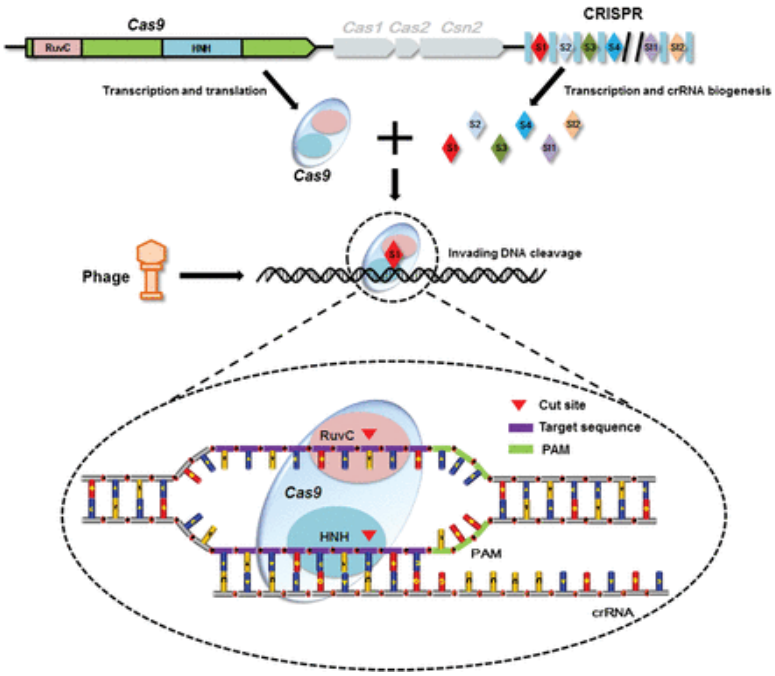

Figure 2: The CRISPR-Cas9 DNA cleavage system. Targeting requires a DNA sequence matching the crRNA and a 3nt PAM sequence downstream of the target. crRNA guides Cas9 to the genomic locus of interest. Nuclease domains in Cas9 create a double stranded break in the target DNA (Zhang et.al. 2014).

In the past, it has been challenging to differentiate between passive participant molecules and crucial components of GLUT4 translocation. However, the advent of CRISPR-Cas9 technology is mitigating this difficulty. CRISPR-Cas9 is revolutionary in its efficiency, specificity, and relative simplicity. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and the associated Cas proteins comprise an endogenous bacterial defense mechanism capable of recognizing and eliminating invading

genetic material (Reis 2014). Three different CRISPR mechanisms have been identified in bacteria, and the type II system is unique in that it requires only one Cas protein, known as Cas9, for gene mutagenesis. Cas9 protein from the bacterium *Streptococcus pyogenes* has been harnessed to create targeted genomic changes in mammalian cells (Ma et.al. 2014). Endogenous Cas9 must complex with CRISPR-RNA (crRNA) and trans-activating crRNA (tracrRNA) for site-specific DNA recognition and cleavage (Ma et.al. 2014). This system has been simplified by combining crRNA and tracrRNA into a synthetic single guide RNA (sgRNA) (Jinek et.al. 2012). A sgRNA homologous to the chosen DNA target allows for site-directed mutagenesis in any gene of interest. After complexing with sgRNA, the RuvC and HNH nuclease domains of Cas9 create a double stranded break in the targeted genomic DNA as shown in Figure 2 (Ma et.al. 2014). This break is repaired by homology-directed repair in the presence of template DNA, or non-homologous end joining (NHEJ) if no DNA is introduced (Overballe-Petersen et.al. 2013). NHEJ is inherently mutagenic, creating indels leading to a frameshift. Frameshift can cause loss of protein function due to altered primary structure, or a premature stop codon. In this way, it is possible to selectively knockout any non-essential gene and evaluate its effect on cell function. Here we did this using a flow cytometry-based assay. This project describes the creation of six knockout cell lines to determine the role of individual genes in GLUT4 trafficking.

III. Materials and Methods

GLUT4 Reporter Cell Lines:

Mouse preadipocyte cells were obtained from Shingo Kajimura at the University of California, San Francisco. Mouse preadipocytes, HeLa cells, and 293T cells were maintained in DMEM supplemented with L-glutamine, 10% FB essence, and penicillin/streptomycin. To differentiate into adipocytes, preadipocytes were cultured to 95% confluence before a differentiation mixture

was added in the following concentrations: 5 µg/mL insulin (no. I0516; Sigma), 1 nM Triiodo-L-thyronine (T3; no. T2877; Sigma), 125 mM indomethacin (no. I-7378; Sigma), 5 µM dexamethasone (no. D1756; Sigma), and .5 nM isobutylmethylxanthine (IMBX; no. 15879; Sigma). After two days, the cells were switched to DMEM supplemented with 10% FB essence, 5 µg/mL insulin, and 1 nM T3. After another two days, the cells were switched to DMEM supplemented with 10% FB essence and 1 nM T3. Differentiated adipocytes were analyzed six days after induction.

HeLa and preadipocyte cell lines expressing the GFP-GLUT4-HA reporter were generated by a graduate student in the lab using lentiviruses containing GFP-GLUT4-HA plasmid. Production of lentiviruses is described in detail below.

CRISPR-Cas9 Genome Editing of Candidate Genes:

To edit a candidate gene, two 20 bp guides were selected within the early constitutive exons of the gene. Oligonucleotides containing one guide sequence were cloned into the pLenti-CRISPR-V2 vector, shown in Figure 3 (no. 52961; Addgene). Oligonucleotides containing the other guide were cloned into the pLenti-CRISPR-V2-Blast vector (no. 83480; Addgene), or a modified version of the pLentiGuide puro, in which the puromycin selection marker was replaced with a hygromycin selection marker (pLentiGuide-Hygro). The resulting plasmids were mixed with U6 primer (Integrated DNA Technologies) and sent to

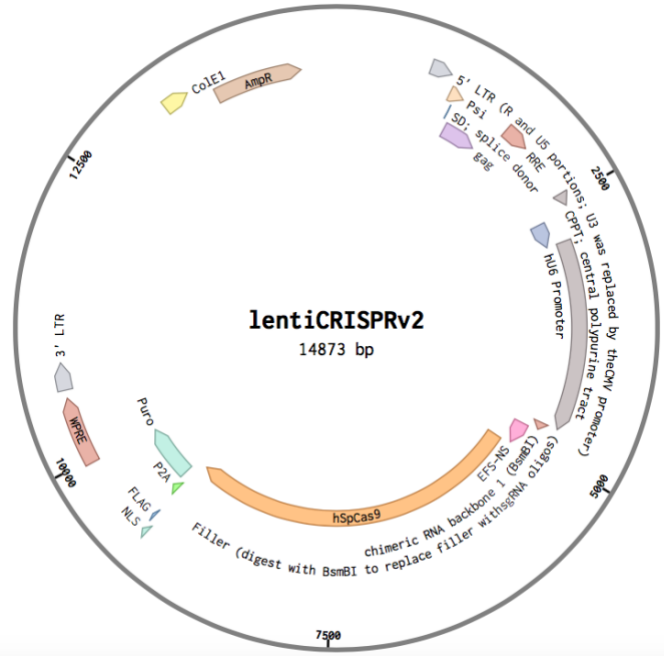

Figure 3: Plasmid map of pLenti-CRISPR-V2 vector (Benchling).

QuintaraBio for sequencing to verify insertion of the guide. Lentiviruses produced from the CRISPR plasmids were used to infect target cells. Infected cells underwent two rounds of selection using 1 μg/mL puromycin, and 500 μg/mL hygromycin or 10 μg/mL blasticidin depending on the vector used. The list of sgRNAs used to edit each gene is given below.

Species	**Gene**	**Target 1**	**Target 2**
Human	EXOC3	CGCCCGGACCAGCTGGACA	CAAAGACATCCAGCAGTCGC
Human	EXOC7	TTCTCCGTCTGCTTGTGCAC	AATCTTGGCCATGCTTCCC
Mouse	OSBPL8	AAAGATGAGTCAGCGCCA	TCTCCCAAAGGTTTTGAAAG
Mouse	OSBPL10	CCAGGACCTGCTTCTCCTGA	CCGCCAGTGCCAACATAACA
Mouse	Tomosyn-1&2	ATCACTGCTGCTCCGCTGTC (tomosyn-1)	CAGTTAAGCCATCCAAAACT (tomosyn-2)

Production of Lentiviruses:

293T cells were grown to 90% confluency and transfected with the pLenti-CRISPR vector along with three packaging plasmids: pAdVAntage (no. E1711; Promega), pCMV-VSVG, and psPax2 using the transfection reagent PEE25. Lentiviral particles were collected 48 hours after transfection and every 24 hours thereafter for a total of four collections. Lentiviruses were pooled and concentrated by centrifugation in a Beckman SW28 rotor at 25,000 rpm for 1.5 hours. Viral pellets were resuspended in PBS and used to infect HeLa cells and preadipocytes.

Flow Cytometry GLUT4 Assay:

Adipocytes and HeLa cells at 100% confluency were washed three times with KRH buffer [121 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 0.33 mM $CaCl_2$, and 12 mM Hepes (pH 7.0)]. After incubation in KRH buffer for two hours, cells were treated with 100 nM insulin for 30 minutes

(cells were treated with 50nM insulin in the tomosyn-1&2 KO experiment). Where appropriate, 100nM wortmannin (no. W1628; Sigma) was added 10 minutes before insulin treatment. After insulin stimulation, cells were chilled in an ice bath and their surface reporter GLUT4 was stained using anti-HA primary antibody (no. 901501; BioLegend) and allophycocyanin (APC)-conjugated secondary antibody (no. 17-4014; eBioscience). The cells were dissociated from the plates using Acutase (no. AT 104; Innovative Cell Technologies), and their APC and GFP fluorescence was measured on a Cyan ADP analyzer (Beckman Coulter, Inc.) Data from populations of ~2,500 cells were analyzed using FlowJo software. To calculate normalized surface levels of the GLUT4 reporter, the mean surface GLUT4 reporter fluorescence (HA-APC) was divided by the mean total GLUT4 reporter fluorescence (GFP). These values were then normalized to those of WT untreated samples. Statistical significance was calculated using a two sample t-test assuming equal variances based on experiments run in biological triplicate.

Flow Cytometry Transferrin Receptor Assay:

Adipocytes at 100% confluency were washed three times with KRH buffer. After incubation in KRH buffer for two hours, cells were chilled and stained for transferrin receptor using anti-CD71 (transferrin receptor) primary antibody (no. 14-0711-82; eBioscience) and allophycocyanin (APC)-conjugated secondary antibody (no. 17-4014; eBioscience). The cells were dissociated from the plates using Acutase (no. AT 104; Innovative Cell Technologies) and their APC fluorescence was measured on a Cyan ADP analyzer (Beckman Coulter, Inc). Data from populations of ~2,500 cells were analyzed using FlowJo software. Mean APC fluorescence was normalized to that of WT samples. Statistical significance was calculated using a two sample t-test assuming equal variances based on experiments run in biological triplicate.

Immunoblotting:

Differentiated adipocytes and HeLa cells grown in 24-well plates were washed 3x with KRH buffer. After incubation in KRH for two hours, where appropriate cells were treated with 100nM insulin for 30 minutes. Cells were then lysed in 2x SDS protein sample buffer and the cell lysates were run on 8% SDS/PAGE gels. Proteins were detected using primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Primary antibodies used were anti-phospho-akt (no. 9271S; Cell Signaling Technology), anti-akt (no. 9272S; Cell Signaling Technology), and anti-α-tubulin (no. 12G10; Developmental Studies Hybridoma Bank).

IV. Results

Genes for this study were chosen through two methods. The first line of inquiry involved the verification of genes identified in two genome wide CRISPR screens in HeLa cells performed by a graduate student in the lab (Gulbranson 2017). One screen sorted for a mutagenized cell population with defects in GLUT4 translocation. The other sorted for a mutagenized cell population demonstrating upregulated surface levels of GLUT4. These screens resulted in a list of candidate genes potentially acting as positive or negative regulators of GLUT4 translocation, respectively. Some of the genes identified were known regulators of GLUT4 translocation, but others were not previously discovered as part of the GLUT4 transport pathway and therefore merit further investigation. Genes were also chosen based on existing literature in the area of insulin signaling and vesicle transport. In order to begin investigating the role of individual genes in GLUT4 trafficking, CRISPR-Cas9 was used to create knockout (KO) cell lines. KO cell lines underwent a flow cytometry-based assay to quantify the levels of GLUT4 surface reporter compared to wild type (WT) cells.

Both HeLa cells and mouse adipocyte cells were used in this study. The aforementioned CRISPR screens were performed in HeLa cells due to their resilience and ease of expansion. While HeLa cells do not express GLUT4 endogenously, it has been shown GLUT4 regulation is conserved between HeLa cells and adipocytes (Gulbranson 2017). HeLa cells are easily maintained and frequently used to study human genetics. While mouse adipocyte cells have a different genetic makeup than human cells, many genes are conserved across the two species and adipocyte cells are more physiologically relevant in the study of insulin-stimulated GLUT4 translocation. Each cell line presents advantages and disadvantages, and both were examined in this study.

Two control HeLa cell lines were created in the first stages of experimentation to ensure the CRISPR-Cas9 machinery functioned as expected. One cell line was exposed to an empty pLenti-CRISPR-V2 vector that did not contain guide RNA (gRNA). Without gRNA, Cas9 should not mutate any gene and therefore there should be no phenotypic difference between cells containing the empty vector (EV) and WT cells. Another cell line was targeted with a CRISPR-Cas9 vector containing gRNA complementary to the Rab10 gene. Rab10 is a known positive regulator of GLUT4 exocytosis, therefore Rab10 knockout should significantly reduce surface levels of GLUT4. As expected, the EV CRISPR construct did not impact GLUT4 translocation (Fig. 5), while the Rab10 KO cell line showed significantly reduced surface levels of the GLUT4 reporter after insulin stimulation (Fig. 4).

To begin examining GLUT4 trafficking, EXOC3 and EXOC7 were knocked out in HeLa cells. EXOC7 appeared as a hit in the translocation defective CRISPR screen, and therefore represents a potential positive regulator of GLUT4 translocation. EXOC3 was not identified as a hit in the screen but has been proposed as a positive regulator of GLUT4 trafficking in previous

literature (Inoue et.al. 2006). EXOC3 and EXOC7 comprise two of the eight subunits in the exocyst complex, a highly conserved multisubunit protein implicated in tethering secretory vesicles to the plasma membrane (Heider et.al. 2012).

Interestingly, knockout of EXOC7 caused a significant phenotype while knockout of EXOC3 did not. EXOC7 KO cells showed reduced surface levels of the GLUT4 reporter under basal conditions, and only showed approximately 1.2-fold increase in the reporter in response to insulin while WT HeLa cells showed 3-fold increase (Fig. 5). In contrast, EXOC3 KO cells showed similar basal levels of the GLUT4 reporter and experienced the same increase in response to insulin as WT HeLa cells (Fig. 4). As outlined in the introduction, the exocyst complex is a known driver of GLUT4 exocytosis. However, based on these preliminary results it appears there is potentially redundancy among the subunits.

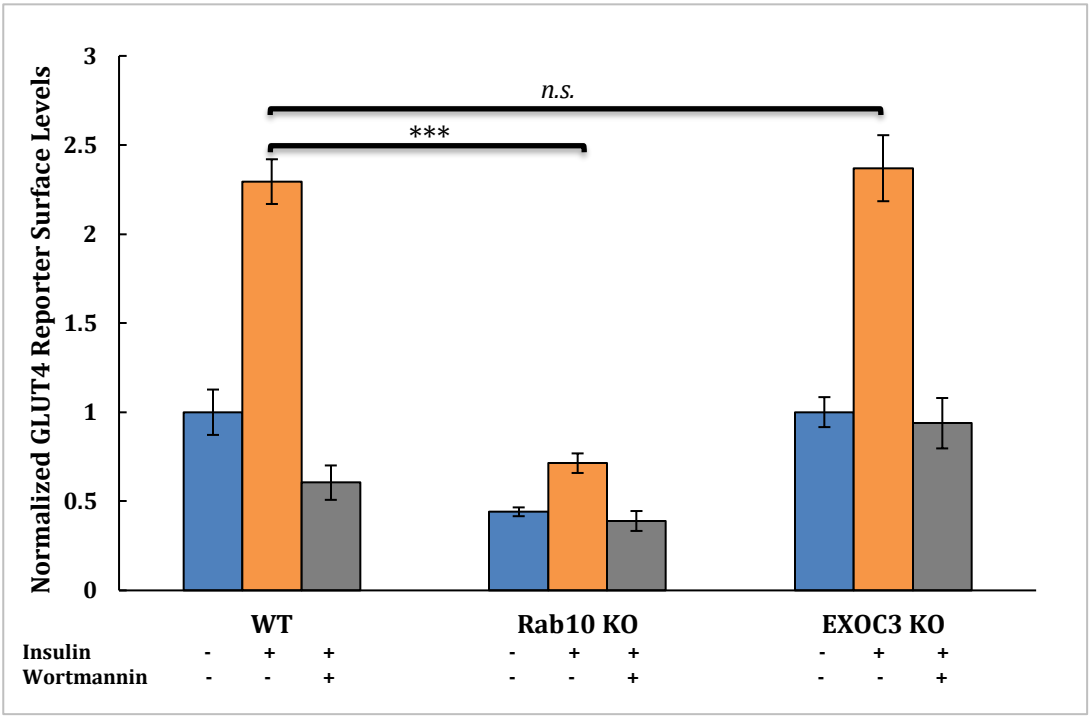

Figure 4: Normalized surface levels of the GLUT4 reporter in the indicated HeLa cells. n.s., not significant. ***P<0.001. Error bars indicate SD. N=3.

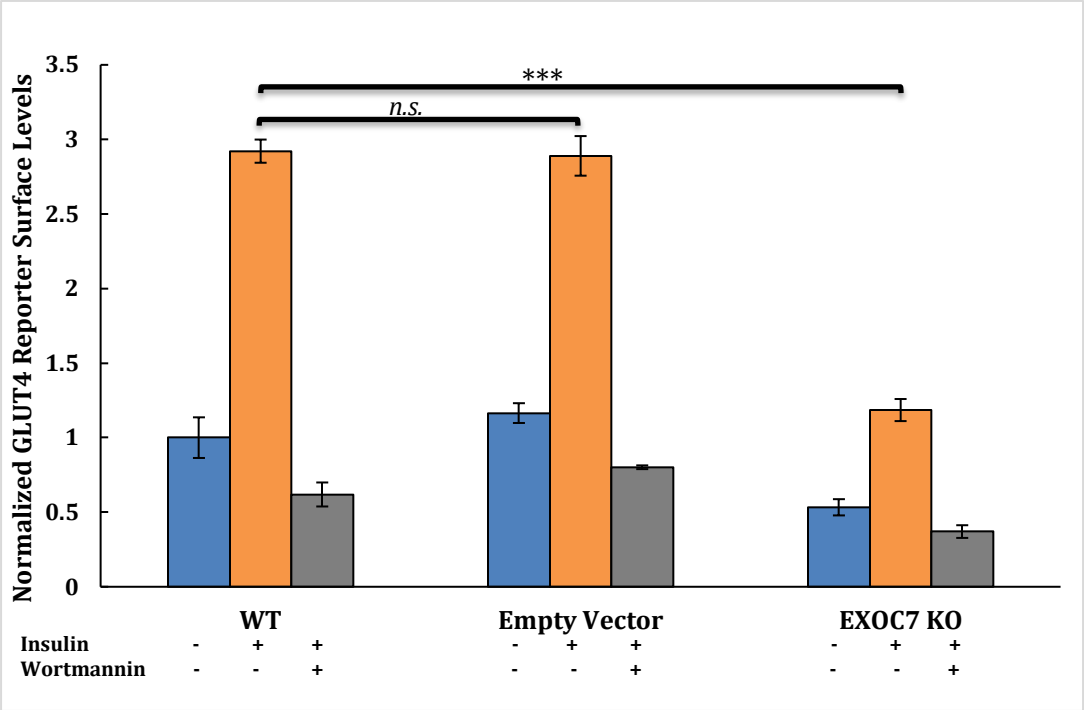

Figure 5: Normalized surface levels of the GLUT4 reporter in the indicated HeLa cells. n.s., not significant. ***P<0.001. Error bars indicate SD. N=3.

To further investigate the mechanism of EXOC7, immunoblotting was performed on lysate from EXOC7 KO cells to assess levels of phospho-AKT (pAKT). The phosphorylation of AKT is a crucial step in propagating the insulin signaling pathway and eventually stimulating GLUT4 exocytosis. EXOC7 KO cells show less total AKT than WT cells (Fig 6). Considering the overall decrease in AKT, EXOC7 does not appear to affect insulin-stimulated phosphorylation of AKT. However, a decrease in total AKT could still hinder insulin signaling as there would be less AKT available for phosphorylation in response to insulin.

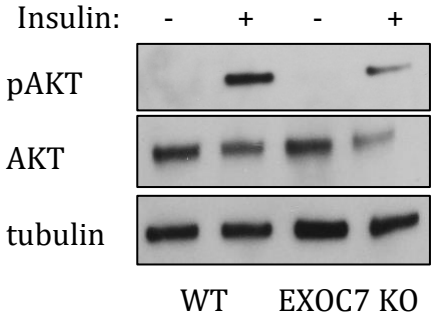

Figure 6: Immunoblots showing total AKT and insulin-stimulated AKT phosphorylation in WT and mutant HeLa cells.

Next, single knockouts of OSBPL8 and OSBPL10 as well as a double knockout of both genes were created in preadipocytes. OSBPL8 and OSBPL10 were identified in the constitutive translocation screen. These two genes encode proteins containing a highly conserved C-terminal oxysterol-binding protein-like sterol-binding domain. Oxysterol-binding protein (OSBP) is a lipid transfer protein, and 12 OSBP-like proteins have been identified in mammals (Olkkonen 2015). OSBPL8 and OSBPL10 have been studied as regulators of lipid metabolism, but their role in GLUT4 trafficking has not been characterized. As shown in Figure 7, OSBPL8 and OSBPL10 appear to act as negative regulators of GLUT4 translocation. OSBPL8 KO, OSBPL10 KO, and double KO cells showed 2-fold higher basal surface levels of the GLUT4 reporter compared to WT adipocytes (Fig 7). In response to insulin, surface levels of the GLUT4 reporter are increased approximately 6-fold in the single KO cells while WT insulin-stimulated cells only

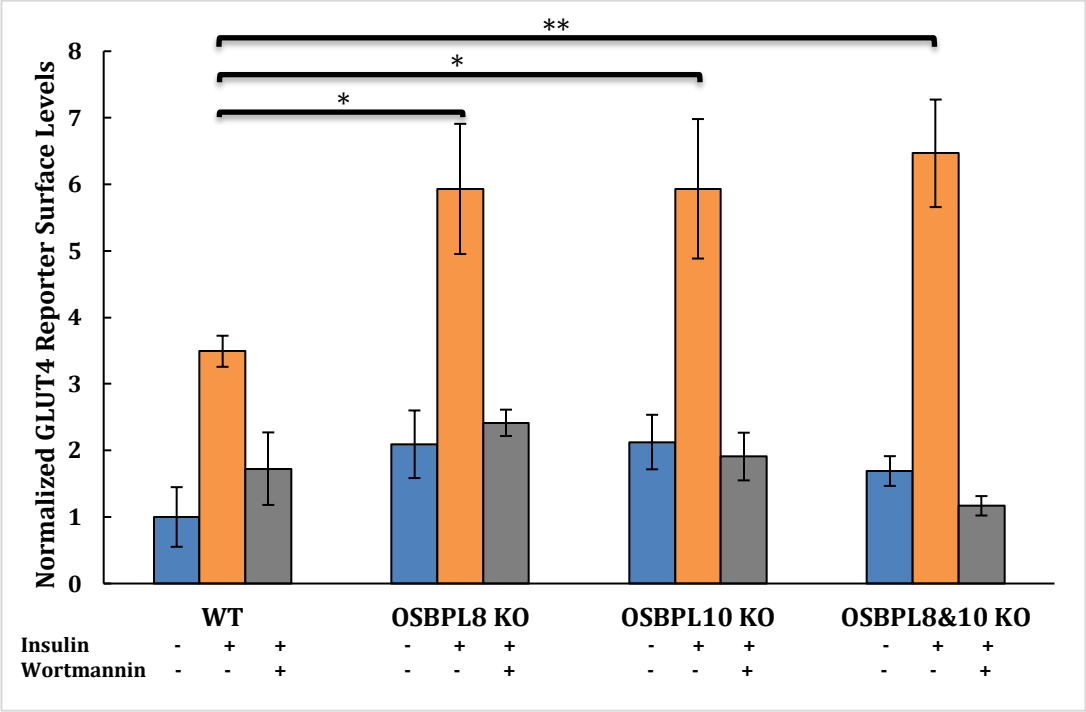

Figure 7: Normalized surface levels of the GLUT4 reporter in the indicated adipocytes. *P<0.05, **P<0.01. Error bars indicate SD. N=3.

show a 3.5-fold increase. Insulin-stimulated double KO cells show even higher surface levels of GLUT4 at approximately 6.5-fold. OSBPL8 KO cells and OSBPL10 KO cells each showed a phenotype in the assay, indicating they have distinct modes of action. The phenotype was slightly more pronounced in double KO cells, but not significant, therefore these genes may have some degree of redundancy.

Immunoblotting showed pAKT levels were higher in insulin-stimulated KO cells compared to WT, suggesting OSBPL8 and OSBPL10 may act to suppress insulin signaling (Fig. 8). OSBPL10 KO cells only show slightly upregulated levels of pAKT, but the phenotype is more pronounced in OSBPL8 KO cells. Double KO cells demonstrate the most distinct increase in pAKT, which supports the findings of the flow cytometry assay and suggests there may be slight redundancy between OSBPL8 and OSBPL10.

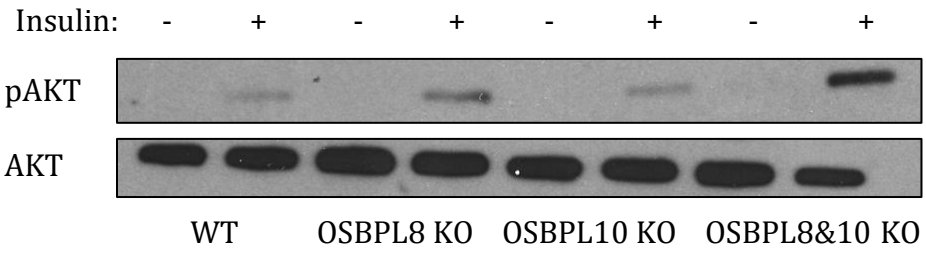

Figure 8: Immunoblots showing total AKT and insulin-stimulated AKT phosphorylation in WT and mutant adipocyte cells.

To determine if OSBPL8 and OSBPL10 act as specific regulators of GLUT4 translocation, a flow cytometry-based assay was performed to quantify surface levels of the transferrin receptor (TfR) in mutated cells. At the plasma membrane, TfR binds and internalizes transferrin, a protein that facilitates iron uptake. TfR undergoes continuous trafficking between the plasma membrane and endosomes as each receptor is recycled to perform several rounds of clathrin-mediated

endocytosis (Mayle et.al. 2012). OSBPL8 KO cells did not show significant difference in TfR surface levels compared to WT cells, but OSBPL10 KO cells showed a significant decrease in TfR surface levels (Fig 9). This supports the findings of the GLUT4 assay, suggesting OSBPL8 and OSBPL10 have distinct functions. While OSBPL8 may act specifically as a negative regulator of GLUT4 trafficking, OSBPL10 appears to be involved in at least two trafficking pathways.

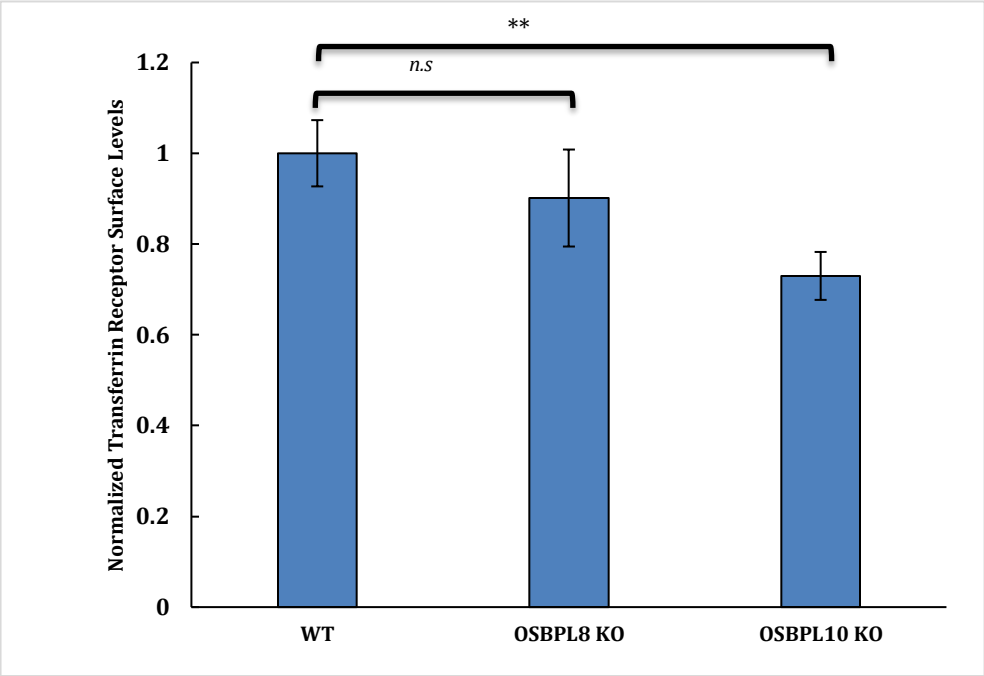

Figure 9: Normalized surface levels of the transferrin receptor in the indicated adipocytes. n.s., not significant. **P<0.01. Error bars indicate SD. N=3.

Finally, a double knockout of tomosyn-1 and tomosyn-2 was created in preadipocytes. Tomosyn-1 and tomosyn-2 are syntaxin-1A-binding proteins and regulate SNARE complex formation (Bhatnagar et.al. 2014). SNARE complex formation is a critical final step in GLUT4 translocation. Tomosyn-1 and tomosyn-2 have been described as inhibitors of exocytosis, but not specifically in relation to GLUT4 translocation. Figure 10 shows knockout of tomosyn-1 and

tomosyn-2 resulted in higher surface levels of the GLUT4 reporter in response to insulin. Surface levels of GLUT4 were increased 6-fold in insulin-stimulated KO cells while WT cells only demonstrated a 3-fold increase (Fig. 10).

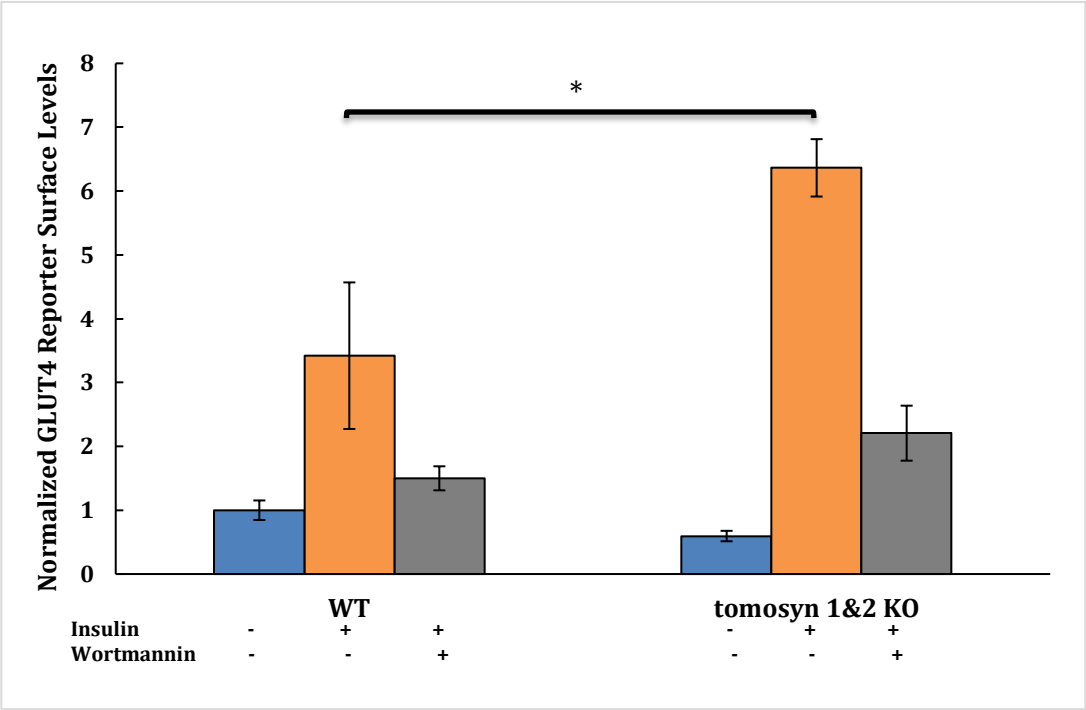

Figure 10: Normalized surface levels of the GLUT4 reporter in the indicated adipocytes. *P<0.05. Error bars indicate SD. N=3.

Immunoblotting showed both pAKT and total AKT levels in tomosyn-1&2 KO cells were similar to WT levels (Fig. 11). Tomosyn-1 and tomosyn-2 appear to act downstream of the phosphorylation of AKT.

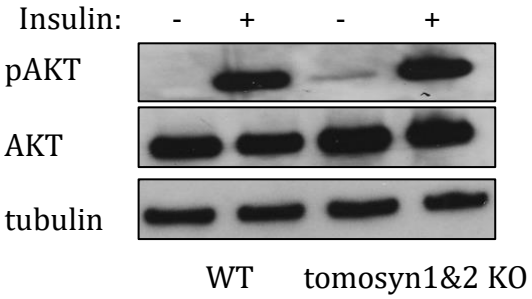

Figure 11: Immunoblots showing total AKT and insulin-stimulated AKT phosphorylation in WT and mutant adipocyte cells.

V. Discussion

Insulin-stimulated GLUT4 translocation relies on an immensely complex molecular mechanism integrating cytoskeletal proteins, components of the insulin signaling pathway, and vesicle transport machinery. GLUT4 trafficking is heavily researched, primarily due to the implications in treating Type II Diabetes, a disease characterized by insulin resistance. Researchers have partially described the GLUT4 transport pathway, but the complete mechanism remains unknown. The goal of this research was to investigate novel regulators of GLUT4 translocation. CRISPR-Cas9 technology was used to ablate six genes and assess the effect on GLUT4 translocation using a flow cytometry-based assay. Candidate genes were chosen based on relevant literature in membrane trafficking and to verify the results of two genome wide CRISPR screens performed by the lab.

In the first part of this study, EXOC3 and EXOC7 were knocked out in HeLa cells. These genes were expected to act as positive regulators of GLUT4 translocation. EXOC7 KO cells demonstrated reduced surface levels of the GLUT4 reporter while EXOC3 KO cells did not show a phenotype (Fig. 4,5). The exocyst complex has been shown to play a critical role in GLUT4 translocation and it appears there is potential redundancy among the subunits where EXOC3 is concerned. Although EXOC7 KO cells demonstrated impaired GLUT4 translocation, EXOC3 KO cells retained functionality, indicating another part of the complex was able to mask the mutation. Immunoblotting showed levels of total AKT were downregulated in EXOC7 KO cells, suggesting EXOC7 KO may impair insulin signaling by reducing the amount of AKT available for phosphorylation (Fig. 6).

To investigate potential negative regulators of GLUT4 translocation, OSBPL8, OSBPL10, tomosyn-1, and tomosyn-2 were knocked out in preadipocyte cells. OSBPL8 KO cells and

OSBPL10 KO cells showed increased surface levels of the GLUT4 reporter, both with and without insulin stimulation. This phenotype was slightly more pronounced in double KO cells (Fig. 7). pAKT levels were noticeably upregulated in OSBPL8 KO cells and double KO cells, indicating these genes may negatively regulate the phosphorylation of AKT in the insulin signaling pathway (Fig. 8). A flow cytometry-based assay showed surface levels of transferrin receptor were downregulated in OSBPL10 KO cells, suggesting OSBPL10 does not only act to regulate GLUT4 trafficking but may play a regulatory role in other trafficking pathways as well (Fig. 9). Tomosyn-1 and tomosyn-2 double KO cells also demonstrated increased surface levels of the GLUT4 reporter in response to insulin (Fig. 10). Levels of total AKT and pAKT were unaffected in tomosyn-1 and tomosyn-2 double KO cells, therefore tomsyn-1&2 likely act downstream of insulin signaling (Fig. 11).

The most significant deficiency in this study is the lack of knockout verification via immunoblotting. The CRISPR-Cas9 mechanism used in this study demonstrates high fidelity, and two rounds of drug selection ensure only cells with the CRISPR vector survive. However, this system is not perfect. Verifying knockouts is ongoing and will be crucial in making definitive conclusions about the role of these genes in GLUT4 trafficking.

This study presents many directions for future investigation. EXOC7, OSBPL8, OSBPL10, tomosyn-1, and tomosyn-2 are interesting subjects for continued research. After verifying knockouts, secondary experiments would concentrate on characterizing the regulatory mechanisms of these genes. A fluorescent antibody-based endocytosis assay could show if OSBPL8 and OSBPL10 are involved in retrieving GLUT4 from the plasma membrane once insulin signaling is terminated. Another useful tool is total internal reflection fluorescence microscopy (TIRFM), which allows for the visualization of events near the plasma membrane.

TIRFM could be used to analyze EXOC7 KO cells and show whether trafficking deficiency lies in translocating GLUT4 to the plasma membrane, or in docking and fusion with the plasma membrane. Co-immunoprecipitation and subsequent mass spectrometry could identify protein interactions and assess whether these proteins are interacting with any known regulators of GLUT4 translocation. This study opens the door to many future projects, and there is a myriad of experiments which can further elucidate the regulatory mechanisms of these genes.

Understanding GLUT4 trafficking at a molecular level is essential to the development of targeted treatments for Type II Diabetes, which is quickly becoming an enormous economic burden. CRISPR-Cas9 technology presents an exciting new means to examine GLUT4 translocation through site-directed mutagenesis of potential regulators. This study contributed six knockout cell lines to the field and represents the first step in characterizing novel molecular players in the GLUT4 trafficking pathway.

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VII. Sources

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