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Double Knockout of Munc18b and Munc18c Reduces Translocation of GLUT4 in Response to Insulin

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March 30th 2016
I. Abstract

Insulin resistance has become a prominent issue in the United States, causing serious comorbid conditions. One crucial step in insulin response in adipose and muscle tissue is the movement of glucose transporter GLUT4 to the cell surface from storage vesicles. This process requires the SNARE complex to drive membrane fusion. Munc18b and Munc18c are syntaxin binding proteins associated with the SNARE complex during fusion. While protein binding assays have revealed interactions of these proteins, their function in GLUT4 translocation has not been well understood. In an effort to establish the role of these proteins in GLUT4 translocation and insulin response, we performed a single knockout experiment in a mouse adipocyte cell line. When insulin mediated GLUT4 translocation did not change significantly, we performed a double knockout experiment. Following double knockout, the ability of these adipocytes to translocate GLUT4 from storage vesicles to the plasma membrane was observed to decrease dramatically. These results indicate an important role for Munc18b and Munc18c in SNARE complex formation and in insulin response.
II. Introduction

Insulin response is a key component of metabolism, allowing cells to regulate blood glucose levels through the uptake of glucose from the blood stream. Insulin is secreted by the beta cells of the pancreas in response to elevated blood glucose levels. When beta cells undergo glycolysis in the presence of high blood glucose, mitochondrial metabolism results in a signaling cascade, triggering the release of insulin (MacDonald et al. 2005). Insulin reduces the concentration of blood glucose in three ways. Firstly, it stimulates muscle and adipose tissues to take up glucose. Secondly, it upregulates glycogenesis in the liver. Finally, it inhibits the secretion of glucagon from pancreatic alpha cells, thus preventing the liver from producing more glucose via glucogenesis (Aronoff et al. 2004). Although adipocyte tissue performs only about 10% of insulin mediated glucose uptake, with muscle tissue performing the remaining 90%, adipocytes are none the less crucial in insulin response (Leto and Saltiel 2012). Not only do adipocytes bring balance by releasing hormones in response to shifts in energy in the body, but inflammation in fat tissue and increased adipocyte size have both been implicated in insulin resistance (Xu et al. 2003, Salans et al. 1968).

Insulin resistance has become a serious health concern. In 2012, diabetes was the 7th leading cause of death in the United States (Centers for Disease Control and Prevention 2015). Approximately 95% of diagnosed diabetics suffer from type 2 diabetes; meaning about 27.6 million Americans are insulin resistant (Centers for Disease Control and Prevention 2014). Diabetes is particularly problematic in that it causes a variety of co-morbid conditions, including hypertension, stroke, heart attack, renal disease, and blindness. Diabetes is the leading cause of amputation in the United States. The disease also creates a financial burden, with diabetics spending an estimated 2.3 times as much as the average citizen on health care. In 2012 diabetes was estimated to have cost the nation $245 billion. This amounts to a 41% increase from 2007’s estimate (American Diabetes Association 2014). Treatments targeted toward insulin resistance have massive potential to drastically improve millions of lives, prevent severe comorbid consequences such as amputation, and reduce the financial burden diabetes creates for individual patients and the nation.

GLUT4 is the primary transporter of glucose in adipose and muscle tissue (Stockli et al. 2011). In the absence of insulin, about 95% of GLUT4 is held within the cell in the trans-Golgi network, endosomes, and GLUT4 storage vesicles (GSVs). When insulin binds its receptor, the now active receptor stimulates phosphoinositide 3 kinase (PI3K) and APS (adapter protein with Pleckstrin homology and Src homology 2 domains) signaling pathways, which regulate the cycling of GLUT4. PI3K is recruited to the plasma membrane and activated by insulin receptor substrate proteins which are phosphorylated by the insulin receptor upon activation of the receptor. PI3K triggers a protein cascade which leads to regulation of small GTPases which are implicated in GSV retention and targeting (Leto and Saltiel 2012). The APS pathway is simultaneously activated when APS is recruited to the plasma membrane by the insulin receptor. Proteins c-CBL and CAP are also recruited. This triggers a cascade that activates TC10, which interacts with the Exocyst complex to create targeting sites for GSVs. Activation of TC10 also leads to the translocation of GAPEX5 to the plasma
membrane. GAPEX5 regulates a number of target GTPases which play a role in GSV retention and translocation. Following recruitment of the GSVs to the plasma membrane, the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex provides a necessary driving force for membrane fusion (Leto and Saltiel 2012). In the presence of insulin binding, the amount of GLUT4 on the cell membrane increases roughly 10 to 30 fold (Stockli et al. 2011). Both clathrin mediated endocytosis and cholesterol dependent endocytosis are used in adipocytes to move GLUT4 back to the interior of the cell (Leto and Saltiel 2012). Failure of GLUT4 to properly translocate to the plasma membrane results in insulin resistance, and an inability to respond to and regulate variations in blood glucose levels (Stockli et al. 2011)

The role of Munc18b and Munc18c in insulin response is not well understood, however it is known that they associate with various syntaxins that contribute to the SNARE complex. The syntaxin family of proteins provides both the force and specificity needed for proper membrane fusion (Teng et al. 2001). In vitro protein binding assays indicate that Munc18b binds syntaxin 1A, syntaxin 2, and syntaxin 3 with strong affinity, as well as binding syntaxin 4 with weaker affinity (Tellam et al. 1997). Munc 18c was found to bind syntaxin 2 and 4 strongly (Tellam et al. 1997, Mueckler 2001). Binding to syntaxin 4 is of interest because this syntaxin is known to interact with the vesicle associated syntaxin VAMP2, which has been shown to be significant in GLUT4 translocation. Additionally, both Munc18b and Munc18c have been shown to translocate to the plasma membrane in response to insulin, similar to the movement of GLUT4 (Tellam et al. 1997). The role of both proteins in insulin response is poorly understood and controversial, with some data suggesting knockout of Munc18c increases

**Figure 1:** APS and PI3K signaling resulting in the translocation of GLUT4 to the plasma membrane. (From Leto and Saltiel 2012)
translocation, some suggesting knockout inhibits translocation, and others suggesting knockout has no effect (Habets et al. 2009, Kanda et al. 2005, Dugani and Klip 2005, Khan et al. 2001, Leto and Saltiel 2012). Similar controversy exists for Munc18b, which has been shown both to be necessary for SNARE complex formation in some studies and in other studies to have no apparent role in translocation (Khan et al. 2001, Lam et al. 2013). Munc18b has been shown to have an important role in immune cells (Ham and Billadeau 2014), but the function of both proteins in insulin response remains unclear. In an effort to better understand the role of these proteins, our group has conducted both single and double knockout cell culture experiments, using fluorescent staining and flow cytometry to determine the effect of double and single knockout on GLUT4 translocation.

III. Methods

The Cell Line

We obtained a line of pre-adipocyte mouse cells from the lab of Dr. Shingo Kajimura at the University of California in San Francisco. Pre-adipocytes were used because a mitotic cell line was required for culture and viral infection. Mouse cells were chosen for their genetic similarity to human cells. An HA tag was attached to the N terminus of GLUT4 and a GFP tag to the C terminus of GLUT4 in the cells using a lentivirus, making it easy to track GLUT4 in the cell. Cells were cultured in a high glucose DMEM media with L-glutamine, 10% FBS, and Penicillin/streptomycin.

CRISPR Construct

CRISPR plasmids were designed to give infected cells an antibiotic resistance gene, a guide sequence targeted to one specific exon, and the gene for the Cas9 nuclease. The Cas9 enzyme uses the guide sequence as a template and creates a double stranded break in any DNA that contains the guide sequence, knocking out the gene which the guide sequence belongs to. The resistance gene allows the use of a selection process which will kill any cells that have not been transfected with the plasmid DNA. The CRISPR plasmids targeted to knockout Munc18b and Munc18c in single knockout were formed by subcloning double stranded oligonucleotides containing guide sequences into a pLentiCRISPR v2 vector, which contains the Cas9 enzyme and a puromycin resistance gene. In the double knockout experiment plasmids targeted to Munc18c were made using a plentiGuide-hygro vector, which allowed for double selection with both puromycin and hygromycin. Resulting plasmids were then transfected into HEK 293T cells, in addition to packaging helper plasmids pcCMV-VSVG, psPAX2, and pAdVantage to create lentiviral particles. 48 hours following transfection, supernatant was harvested from the cell culture, and filtered through a .45µm syringe. The filtered supernatant was centrifuged at 25,000 rpm for 90 minutes in a Beckman SW28 rotor to concentrate the lentiviral particles. Pre-adipocytes were then transduced with the collected virus. Single knockout cells were infected with two viruses targeting either two Munc18b exons, or two Munc18c exons. Double knockout cells were infected with four
viruses, targeting two exons in each protein. Control cells were infected with lentiviral particles containing an empty pLentiCRISPR v2 vector.

The following guide sequences were used in this study with protospacer adjacent motifs (PAMs) underlined:

Munc18c Exon2:
5’-GCCGGAAAGAAGGCGAATGGAAGG-3’

Munc18c Exon3:
5’-GCAAAATGACAGACCTTCTAGAGG-3’

Munc18b Exon 3:
5’-GTTGTAATATGTCAGATACCTGGG-3’

Munc18b Exon 10:
5’-GTTCAGGTATGAGACCACCAGGGG-3’

Selection

Single knockout cells were selected for separately using pyromycin selection. Cells were exposed to 2µg/ml puromycin 24 hours after infection. In double knockout cells, double selection was used to ensure that only pre-adipocytes successfully infected with viruses targeted toward both genes would persist. Selection with 2µg/ml puromycin was conducted first, 48 hours following viral infection with Munc18b targeted viruses. The surviving cells were then infected with Munc18c targeted viruses, and treated with 50µg/ml hygromycin.

Pre-adipocyte differentiation

Pre-adipocytes were cultured to confluence in culture media containing high glucose DMEM with L-glutamine, 10% FBS, and Penicillin/streptomycin. Upon confluence, induction media was added containing insulin, T3 effector, indomethacin, dexamethasone, and IBMX. After two days, the induction media was changed to maintenance media containing insulin and T3. Two days later fresh maintenance media was added.

Western Blot

Immunoblotting was used to determine the success of the CRISPR Munc18c knockout. Knockout adipocytes were run on an 8% SDS-PAGE gel and separated at 20V. A monoclonal-Munc18c antibody was used to stain the membrane. Munc18c was detected using an anti-rabbit secondary antibody produced by Sigma.
T7E1 Assay

A T7E1 assay was used to determine the presence of CRISPR induced mismatched DNA in exons 3 and 10 in the knockout cell line. The T7 endonuclease cleaves mismatched DNA, allowing the detection of CRISPR damaged DNA. Genomic DNA was extracted from wild type adipocytes and knockout adipocytes. Four PCR reactions were prepared, one with wild type genomic DNA and forward and reverse primers for Munc18b Exon 3, one with knockout DNA and exon 3 primers, one with wild type DNA and primers for exon 10, and one with knockout DNA and primers for exon 10. All reactions were run with dNTPS, HF buffer and Phusion polymerase, at the following cycling conditions: 1 cycle of 60 sec at 98°C, followed by 30 cycles of 15 sec at 98°C, 30 sec at 55°C, and 30 sec at 72°C. The PCR product was run on a 1% agarose gel using standard electrophoresis to estimate the concentration. Once concentration was estimated to be over 25 ng/µl, the PCR products were combined with NE buffer and heated to 95°C for 60 sec and then gradually cooled to 85°C at a rate of 2°C per second and then to 25°C at a rate of .3°C per second to form DNA heteroduplexes. The resulting PCR heteroduplexes were digested with the T7 endonuclease for 60 min at 37°C. The digestion was run on agarose as before to detect broken DNA.

Insulin Treatment

The cells were washed 3 times with KRH buffer and serum starved at 37°C for 2 hours. PI3K inhibitor wortmannin was used as a negative control to block insulin response by preventing PI3K signalling. Wortmannin treatment wells were treated with 200nM wortmannin for 10 minutes. Insulin treatment wells were then treated with 100nM insulin for an additional 30 minutes. Control cells were not treated with insulin or wortmannin.

Staining for Flow Cytometry

Following blocking with cold KRH buffer containing 5% FBS, cells were stained with anti HA11.1 antibody to stain surface GLUT4. Cells were then co-stained with APC conjugated anti-mouse secondary antibody. Accutase was used to dissociate the cells in preparation for flow cytometry.

Flow Cytometry

Undifferentiated 3T3L1 cells were run for control. Cells were run in a PBS buffer containing 5% FBS. FlowJo analysis software was used with forward scatter and side scatter plot gate out. GLUT4 was previously tagged with GFP as described above, and surface GLUT4 was identified by the APC stain. The amount of GLUT4 translocation was determined by dividing the amount of APC fluorescence by the amount of GFP fluorescence.
IV. Results

To achieve full knockout, CRISPR lentiviruses were produced to target 2 exons of each gene, for a total of four viruses. In the single knockout experiment, all viruses had a Cas9 gene and puromycin resistance. In the double knockout experiment, viruses targeted to Munc18b contained a resistance gene for puromycin and a Cas9 gene, while viruses targeted to Munc18c contained a hygromycin resistance gene and did not express Cas9. This allowed for single selection in single knockout cells, and double selection in double knockout cells. Selection was carried out after transfection, to ensure that only cells with the desired knockouts would survive.

Following the CRISPR knockout of either Munc18b or Munc18c, differentiation of pre-adipocytes was necessary to accurately observe the role of Munc18b and Munc18c in mature adipocytes. Following differentiation with media containing differentiation factors, the cells matured into white adipocytes with lipid droplets (Figure 2).

![Figure 2: Cells before (A) and after (B) differentiation. The presence of lipid droplets indicates successful differentiation into mature adipocytes.](image)
After adipocytes were successfully differentiated, western blot and T7EI assay were used to confirm knockout. Munc18c knockout cells showed little to no perceptible Munc18c, suggesting the knockout of this gene was successful (Figure 3). Munc18b DNA showed evidence of CRISPR damage, indicating successful knockout (Figure 4).

![Western blot staining for Munc18c. Tubulin is stained as a loading control. The wildtype adipocytes express a large, dark band indicating high expression of Munc18c protein. The knockout cells express little to no visible band, indicating a lack of Munc18c in these cells. This suggests knockout of the Munc18c gene was successful.](image-url)

**Figure 3**: Western blot staining for Munc18c. Tubulin is stained as a loading control. The wildtype adipocytes express a large, dark band indicating high expression of Munc18c protein. The knockout cells express little to no visible band, indicating a lack of Munc18c in these cells. This suggests knockout of the Munc18c gene was successful.
After knockout was successfully confirmed by western blot or T7EI assay, cells were serum starved and insulin treated. Untreated cells represent the level of GLUT4 translocation observed at basal conditions. The cells treated only with insulin have significantly more surface GLUT4 than untreated cells, and reflect the translocation seen in adipocytes that are taking up glucose. Wortmannin treated cells do not respond to insulin with elevated levels of surface GLUT4, as wortmannin binds and inhibits PI3K, an important molecule in insulin signaling. After treatment, cells were stained with a GLUT4 antibody to detect surface GLUT4 and an APC fluorescent antibody which binds the GLUT4 antibody. This provides a means to separate surface GLUT4, which would emit both a GFP fluorescence and APC fluorescence, from internal GLUT4 which would only emit GFP fluorescence. Thus, the amount of APC fluorescence can be quantified as the ratio of APC:GFP fluorescence to render the percent of GLUT4 that has been translocated to the plasma membrane in each group of cells. Flow cytometry data for the single knockout experiment showed no significant difference in GLUT4 translocation from wild type cells, suggesting that single knockout of Munc18b or Munc18c is not sufficient to impair the cell’s ability to translocate GLUT4 in response to insulin (Table 1, Figure 5).
Table 1: Flow cytometry data comparing wild type (WT) and knockout cells (KO). GFP fluorescence is recorded as total GLUT4, APC fluorescence is recorded as surface GLUT4. Translocation is measured as the ratio of surface GLUT4 to the total amount of GLUT4. Translocation is normalized to WT untreated values. Standard deviation values represent the standard deviation for the translocation column.
Figure 5: Knockout of Munc18b or Munc18c. Wild type (WT), Munc18b, and Munc18c knockout (KO) adipocytes in untreated, insulin, or insulin with wortmannin conditions. Flow cytometry was used to detect GFP and APC fluorescence. The ratio of APC:GFP was computed to determine the percentage of surface GLUT4, then values were normalized to the untreated WT. Asterisk indicates significance at the p < .05, as calculated using non-equivalent two-tailed T-test on Prism software. While there is some statistical significance between the untreated wild type cells and untreated Munc18b knockout cells, and the wortmannin treated wild type and Munc18c cells, we do not believe this to be biologically significant.
Interestingly, single knockout of Munc18b or Munc18c did not cause a significant change in GLUT4 translocation with insulin treatment. We hypothesized that the presence of one protein might be able to compensate for the lack of the other, and preserve insulin response. To test this, we created a Munc18b, Munc18c double knockout adipocyte cell line. We infected pre-adipocytes with four viruses, two targeting Munc18b and two targeting Munc18c, to achieve a double knockout. After double selection and confirmation of the absence of both proteins by western blot and T7EI assay, the double knockout line was differentiated, treated, and stained as before. Flow cytometry data showed that there was a significant difference between the level of GLUT4 translocation in double knockout cells and wild type cells upon insulin treatment, which indicates that both proteins play an important role in proper insulin response (Table 2, Figure 6). This data supports our hypothesis that the proteins are involved in GLUT4 translocation, and that the function of one can compensate for the absence of the other in insulin response.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total GLUT4</th>
<th>Surface GLUT4</th>
<th>Translocation</th>
<th>Normalized Translocation</th>
<th>Stand. Dev.</th>
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<td><strong>WT</strong></td>
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<td></td>
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<tr>
<td>Untreated</td>
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<td>28</td>
<td>0.0396</td>
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<tr>
<td>Insulin</td>
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<td>0.3121</td>
<td>7.8752</td>
<td>0.1307</td>
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<td>9.1</td>
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<td>0.3717</td>
<td>0.0048</td>
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<tr>
<td><strong>Double KO</strong></td>
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<td><strong>29.1</strong></td>
<td><strong>0.0445</strong></td>
<td><strong>1.1233</strong></td>
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<tr>
<td>Insulin</td>
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<td>0.0437</td>
<td>1.1027</td>
<td>0.0455</td>
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**Table 2:** Flow cytometry data comparing wild type and double knockout cells. Translocation is normalized to WT untreated values. Standard deviation values represent the standard deviation for the translocation column.
Figure 6: Flow cytometry data comparing double knockout cells to wild type. Wild type cells have significantly higher translocation than knockout cells when exposed to insulin, indicating that knockout of both Munc18b and Munc18c substantially reduces the ability of the cell to move GLUT4 in response to insulin. Asterisk indicates significance at the p < .05, as calculated using non-equivalent two-tailed T-test on Prism software.
V. Discussion

Although single knockout of Munc18b or Munc18c does not appear to affect the ability of adipocytes to translocate GLUT4 in culture, double knockout of both proteins causes a significant reduction in insulin mediated GLUT4 translocation. The differences in insulin mediated translocation in single knockout cells were not significant (Figure 5). Although there was some unexpected significance between the wortmannin treated wild type and Munc18c knockout cells, we do not believe that Munc18c has any implication in interfering with wortmannin or influencing PI3K signaling. Rather, we believe this difference, and the difference observed between Munc18b knockout untreated and wild type untreated cells are not biologically relevant, and may not be significant after replicate experiments are performed. The data collected for double knockout however is quite compelling, indicating a dramatic decrease in insulin mediated GLUT4 translocation (Figure 6). Not only does this suggest that both proteins have a role in binding syntaxins required for insulin response and aiding in the formation of the SNARE complex, but it also lends credibility to the possibility that the two proteins serve much the same function in GLUT4 translocation, given their apparent ability to substitute for each other.

Now that we have shown that Munc18b and Munc18c are crucial for insulin response, the next step is to perform a wild type rescue. Should the cells regain the ability to translocate GLUT4 in response to insulin, this will further support our current data and confirm that these proteins merit more investigation as potential drug targets. A further step would be to conduct a double knockout and wildtype rescue \textit{in vivo}. Mice would be a logical animal model, given their genetic similarity to humans, and the current knowledge of these proteins in mice. Additionally, knockout mice could be compared to current models of insulin resistance that already exist for mice.

It may also be fruitful to determine whether single knockout of either Munc18b or Munc18c can cause insulin resistance in mice. While single knockout causes no apparent change in insulin response in culture, it is possible that these mice might show symptoms of insulin resistance as they age, especially when subjected to stressors such as a high fat or sugar diet. It is possible that one protein would no longer be able to compensate for the loss of the other during such a challenge condition. Mice heterozygous for one protein might also be examined. In addition to further studies in adipose tissue, similar cell culture experiments could be carried out in muscle tissue, given the important role muscle tissue plays in glucose uptake.

While many experiments must be conducted to determine whether Munc18b/c target therapies would be fruitful, the results presented here demonstrate that Munc18b and Munc18c have a significant role in insulin response. This data illuminates the function of two proteins that have previously been controversial, and helps us to further understand GLUT4 translocation and insulin response.
References


