Target of GLUT4 Regulating Protein TUG-UBL1 to Treat Insulin Resistant Type Two Diabetes

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Target of GLUT4 Regulating Protein
TUG-UBL1 to Treat Insulin Resistant Type Two Diabetes

By

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College of Saint Benedict

CAPSTONE THESIS

Submitted in partial fulfillment of the requirements for the Chemistry Major at The College of Saint Benedict | Saint John’s University, Saint Joseph & Collegeville, Minnesota.

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Abstract

Type two diabetes mellitus is a highly prevalent disease for which the mechanism of insulin signaling to initiate glucose uptake in cleaving TUG-UBL1 by the protease Usp25m is currently not well understood. As increased insulin resistance can lead to the development of type one insulin deficient diabetes, the knowledge of this pathway may uncover underlying causes of insulin resistance. This research aims to understand the effect and stoichiometry of Usp25m binding to TUG-UBL1 in relationship to the cleavage of TUG-UBL1 initiating translocation of GLUT4 glucose transporter. The action of protease effector PIST will be investigated to understand the importance of order in protein presence to initiate TUG-UBL1 cleavage. The mechanism of PIST action on TUG-UBL1 Usp25m protease will be elucidated to understand the cascade of insulin signaling effects on glucose uptake. The understanding of this mechanism will allow for investigation of this mechanism in relationship to a novel therapeutic pathway in insulin resistant diabetes treatment. Current diabetes medications do not directly impact the cellular uptake of glucose but negate the negative symptoms of insulin resistance, while a therapeutic targeting this cleavage pathway can bypass insulin stimulation to reduce increased insulin resistance. The specific description of this mechanism allows for therapeutic small molecule investigations to stimulate or replace PIST action on Usp25m to artificially trigger glucose uptake for diabetes treatment.
Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>4</td>
</tr>
<tr>
<td>Background</td>
<td>5</td>
</tr>
<tr>
<td>Persistence of Diabetes</td>
<td>5</td>
</tr>
<tr>
<td>Glucose as a Source of Energy and Carbon Fragments</td>
<td>7</td>
</tr>
<tr>
<td>Insulin as a Hormone</td>
<td>8</td>
</tr>
<tr>
<td>Current Treatments</td>
<td>9</td>
</tr>
<tr>
<td>Mechanism of Insulin Stimulation</td>
<td>12</td>
</tr>
<tr>
<td>Failure of Insulin Response in Type Two Diabetes</td>
<td>14</td>
</tr>
<tr>
<td>Proposal</td>
<td>17</td>
</tr>
<tr>
<td>Analysis of Usp25m Concentration Necessary to Bind TUG-UBL1</td>
<td>18</td>
</tr>
<tr>
<td>Investigation of PIST activity on Usp25m to Regulate TUG-UBL1 Cleavage</td>
<td>20</td>
</tr>
<tr>
<td>References</td>
<td>22</td>
</tr>
</tbody>
</table>

Figure 1. Diabetes prevalence in the US from 1980-2014 ............................................. 5
Figure 2. Blood sugar levels in hypoglycemia and hyperglycemia ..................................... 6
Figure 3. Use of glucose in glycolysis .................................................................................. 7
Figure 4. Simplified insulin signaling pathway ..................................................................... 7
Figure 5. Insulin pathway through the body ........................................................................... 8
Figure 6. Sulfonylurea medication interaction with pancreatic cells ................................... 8
Figure 7. Metformin target of AMPK ....................................................................................... 10
Figure 8. SGLT2 inhibition of glucose reabsorption ............................................................... 11
Figure 9. Insulin stimulation phosphorylation pathway leading to GLUT4 translocation ...... 12
Figure 10. TUG cleavage with Usp25m .................................................................................... 13
Figure 11. TUG-UBL1 protein with N and C termini ................................................................. 15
Figure 12. Anticipated MALDI/MS peaks from cleavage products .......................................... 17
Figure 13. Experimental scheme of Usp25m concentration determination ................................ 19
Figure 14. Experimental scheme of PIST interaction analysis ............................................... 20
Figure 15. SDS-PAGE gel monitoring of cleavage products in TUG ....................................... 21
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Background

Prevalence Of Diabetes

Diabetes is a disease that has plagued the American population with increasing prevalence in recent years. Type two diabetes mellitus is one of the most imperative medical challenges of the 21st century due to the overconsumption of relatively inexpensive, calorie dense, and highly palatable food that is not satiating. This overconsumption has led to unprecedented increases in obesity which is marked by the combined prevalence of diabetes and prediabetes at 50% of the population in the United States,13 where obesity related type two diabetes mellitus (DMII) accounts for 90-95% of diagnosed diabetes in adults.14 This increased prevalence in the US population places youth at high risk of diabetes diagnosis and development of health problems as diabetes is a lifelong illness which results in long-lasting side effects if not properly managed.

The lifelong impacts of diabetes include hyperglycemia which is marked by the excessive glucose concentration in blood. Hyperglycemia is medically classified as increased blood glucose concentration from the baseline concentration and often leads to long term damage, dysfunction, and failure of organs especially in the eyes, kidneys, nerves, heart, and blood vessels15, 16 which can lead to severe health conditions. Hyperglycemia is associated with symptoms of extreme thirst, frequent urination, tiredness, nausea, and dizziness though extremely high blood sugar levels have the potential to induce confusion or loss of consciousness leading many patients into a diabetic coma. Further caution is required than the monitoring of hyperglycemia alone, as excess insulin or insufficient blood glucose concentration can cause hypoglycemia. Hypoglycemia is indicated by symptoms of racing pulse, cold sweats, pale appearance of the face, severe headache, shivering or feeling weak at the knees, restlessness or difficulty concentration and can be serious if sugar levels drop substantially.6, 17

The symptoms of hyper and hypoglycemia are some of the first indications of diabetes and in many cases the lack of immediate treatment causes severe health impacts.

Figure 1. Patients diagnosed with diabetes in America from 1980-2014 with increasing prevalence.5
Diabetes is classified in two categories of type one and type two diabetes where type two diabetes is also called diabetes mellitus type two (DMII). Type one is classified by the inability to produce insulin, while DMII is metabolic resistance to insulin action. In comparison to type one diabetes which requires supplementary insulin treatment, DMII doesn’t strictly necessitate the addition of insulin, but rather close regulation of blood sugar levels as insulin response is reduced or defective in this type of patient. This resistance insulin results in any insulin present in the blood not decreasing blood glucose levels in the anticipated amount from cellular glucose uptake. Once insulin sensitization is limited to a certain extent, the release or supplementation of insulin becomes ineffective at increasing cellular glucose uptake and symptoms and signs of hypoinsulinism and hyperglycemia appear, which are clinically recognized as diabetes mellitus. Diabetes mellitus is defined as insulin in the body having no appreciable affect and cells acting in pathways that indicate the lack of insulin, therefore exhibiting signs of hypoinsulinism. As such, differentiation of type one and type two diabetes stems from the ability of the pancreas to produce insulin. Type one diabetic patients are physically unable to secrete insulin though they are responsive to supplemental insulin, while DMII patients’ symptoms are due to inadequate response when the cells are stimulated by insulin. Reduced insulin response or sensitivity can lead to several health complications as insulin regulates the absorption of glucose from the bloodstream to the cell to convert sugar into cellular energy. When glucose is not adequately absorbed, blood glucose levels increase and result in symptoms previously discussed due to the cells behaving in a starved state, where carbohydrate energy is not available, and the cells must turn to catabolic processes to supplement the energy needed for cell survival. DMII typically stems from obesity, though it can also be induced by the failure of glucose metabolism, hypercholesteremia, hypertriglyceridemia, and arterial hypertension as well as inherited genetic factors. As insulin is not an effective long-term treatment for patients with DMII, alternative methods must be investigated in regulating blood sugar levels for individuals who do not respond to insulin.

Figure 2. Glucose concentrations from high to low blood sugar indicating hyperglycemia and hypoglycemia respectively. Corresponding glucose concentrations relative to high or low glucose levels although definitive barriers between levels do not exist.
Glucose as A Source of Energy and Carbon Fragments

The primary role of glucose in the body is as an energy and carbon source for metabolism of several important biomolecules. Glucose which enters the cell via GLUT4 transport proteins has two possible fates, glycolysis as shown in figure 3, or glycogen synthesis. The principal pathway of the two fates is glycogen synthesis to store energy for later use which represents 75% of glucose that enters the cell. This proportion of glucose subject to glycogen synthesis supports the role of insulin as an energy storage hormone as glycogen synthesis is activated by insulin stimulation as a central method to decrease blood glucose concentration. The energy stored and produced by glucose is essential in all cell functions as glycolysis initiates the production of pyruvate which is used in many cell energy pathways such as the citric acid cycle and supports the cellular growth and function of all cells in the body. The carbon fragments produced from glycolysis are key regulators of many metabolic pathways, and contribute to the synthesis of nucleotides, lipids, and amino acids, as well as energy in the form of ATP. This synthesis stems from the carbon fragments made throughout the TCA cycle and energy made available for cellular use in the electron transport chain. Without glycolysis, the cell is starved of necessary carbons to use for other biologically necessary pathways such as

Figure 3. Glucose use in the cell to make nucleotides, amino acids, and lipids prior to energy harvesting which takes place in the electron transport chain.9

Figure 4. Insulin signaling by binding to the insulin receptor subunit initiating a phosphorylation cascade resulting in GLUT4 allowing for transport of glucose.8
duplicating the genome utilizing nucleotides, and protein production utilizing amino acids. The effect of insulin on adipose and muscle cells is initiated by the binding of insulin to the insulin receptor subunit which causes a phosphorylation pathway to translocate a glucose transporter protein GLUT4. GLUT4 is sequestered in glucose storage vesicles (GSVs) until insulin stimulation is present. The translocation of GLUT4 allows for selective uptake of glucose dependent on cellular needs. When insulin does not initiate the translocation of GLUT4, glucose is unable to enter the cell for use in the biologically important process of producing energy and small carbon fragments. Stimulation of this highly regulated metabolic process of glucose uptake occurs by insulin binding to the insulin receptor on the membrane of adipose and muscle cells as shown in figure 4. The reception of insulin causing signals across the cell membrane is a key mechanism in the intensity and duration in insulin signaling and is commonly a major disfunction found to cause DMII. This mechanism is a key element in allowing for glucose transport into the cell which is mediated by GLUT4 transporters as seen in figure 4.

**Insulin as a Hormone**

In contrast to most hormones which are small biomolecules functioning to regulate cellular pathways, insulin is a polypeptide hormone controlling lipid and glucose metabolism produced by the pancreas. To build this polypeptide hormone necessary to signal glucose uptake, insulin is produced by beta cells in the pancreas regulated by glucose, amino acid, keto acid, and fatty acid concentrations. This insulin is then secreted directly to the portal vein of the liver where up to 80% of insulin is cleared to the bloodstream upon first passage to be delivered by action of the heart. The liver controls the output of insulin to the bloodstream where it interacts with the adipose and skeletal muscle cells as shown in figure 5 with the general mechanism described in figure 4. Insulin regulates blood glucose levels via the Na\(^+\)-dependent and Na\(^+\)-independent glucose transporters, where only Na\(^+\)-independent transporters can be affected by insulin stimulation. Na\(^+\)-dependent transporters are present in several tissues such as epithelium and kidney cells where they act in cotransport to absorb glucose by coupling movement to Na\(^+\) into the cell. Thus, this process is dependent on extracellular and intracellular ion concentrations which are maintained by the Na\(^+\)/K\(^+\) ATPase ion pump. In contrast, the Na\(^+\)-independent glucose transporter
family facilitates the movement of glucose down the concentration gradient across the plasma membrane. Seven isoforms have been identified (GLUT1-7) although GLUT4 is the transporter with highest concentration in insulin-sensitive tissues such as adipose and muscle cell tissues. GLUT4 is the main glucose transport protein utilized in the insulin stimulation pathway to induce glucose entry into the cell for use in energy producing and storage processes. After use in adipose and skeletal muscle, insulin travels through the bloodstream to the kidney to be excreted.

**Current Treatments**

The deficiency in the uptake of glucose into the cell is the primary marker of DMII, as previously discussed, and has been studied extensively. Insulin resistance can be clinically determined with glucose and insulin injections while monitoring for blood glucose levels. The magnitude that injected insulin suppresses hyperglycemia is monitored as lasting hyperglycemia post introduction of insulin indicates that insulin is making little to no effect on glucose uptake on the cellular level. When insulin is observed to not reduce blood sugar levels, this drastically changes the treatment methodology from simply injecting insulin to the monitoring of blood glucose levels. This analysis of insulin effect on blood glucose levels is highly essential to differentiate between type one and type two diabetes. This differentiation is imperative as DMII requires vastly different treatment from type one diabetes, as type one diabetes necessitates supplemental insulin which is far less effective in treating DMII.

In treatment for insulin resistant DMII, many medications currently exist to assist with the metabolic disfunctions caused by insulin resistance. One of the main mechanisms targeted is to promote additional insulin secretion from the pancreas, commonly found in sulfonylurea medications such as glimepiride and glipizide. Other medication mechanisms include increasing insulin sensitivity such as in metformin, or binding to glycose to promote excretion through the urine such as in Jardiance® and Farxiga®. Several combined delivery medications also exist, utilizing two or more of these mechanisms to decrease diabetes related symptoms and improve patient outcomes and quality of life.

Glipizide and glimepiride work similarly, as both are in the class of sulfonylureas which increase the amount of insulin molecules secreted. This is done by stimulating the ATP sensitive potassium channel in the pancreas to induce partial closure of the channel with role shown in figure 6. Sulfonylurea type pharmaceuticals are typically used when pancreatic damage is not yet present due to progressed diabetes mellitus symptoms. This is

![Figure 6. ATP-dependent potassium channel regulation of insulin secretion in interaction with sulfonylureas such as glimpiride and glipizide which open the K<sub>ATP</sub> Channel.](image)
shown in figure 6 depicting the action of ATP sensitive potassium channels in their regulatory role of insulin release. Regulation occurs by the stimulation of this ion channel to its open form; this stimulation facilitates increased amounts of insulin to be released to the bloodstream. Sulfonylureas are extremely short lived and require dosing before every meal. The frequent dosing is combined with side effects such as weight gain and hypoglycemia due to increased secretion of insulin that is not dependent on cellular conditions.

Both dosing regularity and side effects contribute to downfalls of this type of metabolic regulation of glucose metabolism, while this medication also does not specifically solve the disfunction of insulin stimulation not leading to glucose uptake.

Utilizing a different mechanism, altering carbon use in diabetic patients is performed by medications in the biguanide class of which only metformin is approved for patient use. Metformin targets the AMP dependent Protein Kinase (AMPK), where activation inhibits the ATP consuming pathways of fatty acid synthesis and instead promotes ATP synthesis. This artificial low energy state drives the insulin sensitive pathway towards glycolysis, and leads to reduced blood glucose levels as glucose, although the exact mechanism remains controversial. Metformin enters the cell and mimics increased AMP concentration which activates AMPK, therefore decreasing the catabolic gluconeogenesis pathway, and increasing glycolysis to remove glucose from the bloodstream. Activation of AMPK is regularly signaled by the decrease of cellular energy, marked by increased AMP concentrations. This decrease of energy directs the cell by kinase activity towards increased glucose uptake, fatty acid oxidation, and reduced gluconeogenesis as seen in figure 7. The primary effect of this target is in hepatic liver cells which decrease systemic blood glucose levels by the amount of glucose reabsorbed into the bloodstream. Lactic acidosis is possible especially in patients who have liver disease, heart failure, kidney disease, sepsis, or alcohol abuse.4

Jardiance and Farxiga target yet another pathway of decreasing diabetes mellitus effects on the body, as both are inhibitors of sodium-glucose co-transporter 2 (SGLT2) which is responsible for glucose reabsorption by the kidney as seen in figure 8. Inhibiting this reuptake pathway leads to preferential excretion of glucose in the kidney through urine which then decreases the amount of glucose in the bloodstream.14, 25 However, this method does not increase energy production in patients on this medication and basis of treatment is on mitigation of hypoglycemia rather than in allowing cells to uptake glucose. These treatments are typically reserved for patients who are not
currently meeting glycemic target levels with side effects such as genitourinary infection, hypotension, and dehydration.

Even after a patient has been diagnosed with diabetes mellitus and several treatment options are available for use, only about half of patients achieve recommended glycemic targets\textsuperscript{14} which emphasizes the necessity of medications with increased efficacy in reaching glycemic target concentrations, especially in pathways which allow the cell to utilize the energy found in glucose. The lack of medications effective at reaching glycemic concentrations which are desirable while bypassing the deficiencies in DMII metabolism, lead to the necessity of diabetes medications which can act in place of insulin sensitization and promote the usage of glucose as if insulin resistance was not present.

There is an overwhelming need for more effective and specific medications, especially as current medications are focused on symptom-mitigation based solutions rather than circumventing insulin stimulation. The major pathways utilized currently by pharmaceuticals include increasing insulin sensitivity such as in metformin, although off target effects are likely when the target kinase is found throughout the body. Other pharmaceutical treatments allow for increased elimination of glucose, which prevent the additional buildup of side products which induce health effects. However, these pathways do not mitigate the dysfunction in insulin signaling found in diabetes type two, as seen in SGLT2 inhibitors like Jardiance and Farxiga, as well as K\textsubscript{ATP} channel activators such as glipizide and gliclazide. The focus of these medication pathways is on increasing glucose clearance which increases the danger of induced hypoglycemia and hypotension. The ability to use rational design of pharmaceuticals necessitates highly specific interaction of a medication with only the insulin stimulation pathway in a way that could not induce further damage due to overproduction of insulin or negate the cell’s ability to uptake glucose. The possibility of circumventing the requirement of insulin to uptake glucose would be highly beneficial for patients who have progressed insulin resistance with or without damaged β-cells.

A newly developed pharmaceutical with high specificity to the insulin signaling pathway has the possibility to bypass side effects due to off target interactions. Additionally, a therapeutic with this degree of specificity maintains the ability to bypass insulin stimulation, which mitigates the risk of further insulin resistance, while additionally allowing the cell to utilize energy available in the form of glucose found in the blood.

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Figure 8. Sodium-glucose co-transporter 2 (SGLT2) inhibitor pathway to block glucose reabsorption into the blood by regulating kidney clearance.\textsuperscript{7} This mechanism is utilized by SGLT2 inhibitors such as Jardiance and Farxiga.


**Mechanism of Insulin Stimulation**

Essential mechanisms in the insulin stimulation pathway are most notably PI3K (phosphoinositide-3-kinase), which initiates the phosphorylation of PIP2 (phosphatidylinositol-4,5-biphosphate) to PIP3 (phosphatidylinositol-3,4,5-triphosphate). This phosphorylation is upstream to the signaling pathway which releases GLUT4 from sequestration and allows for glucose uptake as seen in figure 9. Experimental disabling of this pathway removes insulin stimulation effects, which supports the idea that PI3K is an essential node in insulin stimulation. This is as PDK1 (phosphoinositidene dependent kinase 1) is affected by the production of PIP3 and Akt is one of the most common readouts associated with successful insulin stimulation. Pharmacological activity that does not require the use of PI3K has great potential to negate the metabolic dysfunction found in DMII, and could be a successful replacement or supplement of insulin injections. The necessity to bypass PI3K indicates the advantage of targeting TUG-UBL1, as this protein is downstream of PI3K action. Additionally, AMPK (AMP activated protein kinase) is a currently targeted kinase in diabetes medication, which has uses in many metabolic processes that could be disturbed with activation of energy building pathways to favor energy utilizing pathways. This disruption has many implications in biological system feedback of ATP concentrations and the body’s need for energy.

In the pathway of insulin stimulation, insulin initiates the cleavage of a Tether containing UBX domain for GLUT4-Ubiquitin like 1 (TUG-UBL1) through a series of second metabolites which promotes GLUT4, a glucose transporter, translocating to the cell surface which enhances glucose uptake. Stimulation from insulin causes TUG-UBL1 to release GLUT4 molecules from within a GSV (GLUT4 storage vesicle). GSVs are tethered to TUG-UBL1 utilizing IRAP (insulin responsive aminopeptidase) to assist location of GLUT4 inside GSV on the N-terminal region as shown in Figure 9 in the far-left cluster. Release of GLUT4 occurs by cleavage of the N-terminal region from the C-terminal region of TUG-UBL1 which induces uptake of glucose through the

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*Figure 9. Insulin signaling secondary messenger pathway. TUG; Tether containing UBX domain for GLUT4, IRS; insulin receptor subunit, PI3K; phosphoinositide-3-kinase, PIP3; phosphatidylinositol-3,4,5-triphosphate, PIP2; phosphatidylinositol-4,5-biphosphate, PDK1; phosphoinositidene dependent kinase 1, AMPK; AMP activated protein kinase GAP; Rab GTPase Activating Protein, IRAP; insulin responsive aminopeptidase, PIST: syntaxin-6-interacting protein, USP25m; protease cleaving TUG, GSV; glucose storage vesicle. Adapted*
GLUT4 transporter. This cleavage of TUG-UBL1 stimulates the release of GLUT4, a glucose transport protein, to the cellular membrane from GSVs in the golgi matrix and allows for the uptake of glucose.26

As shown in Figure 9, the process of GLUT4 translocation is initiated by the binding of insulin to the insulin receptor subunit (IRS). IRS is comprised of two extracellular α-subunits binding to insulin, and two membrane spanning β-subunits that each contain a tyrosine kinase domain that allows initiation of second messenger signaling. The binding of insulin to the α-subunit initiates the phosphorylation of tyrosine residues in the β-subunit which recruits P13K heterodimer and initiates a phosphorylation cascade of second messenger signaling. The P13K heterodimer catalyzes the phosphorylation of PIP3 from PIP2.13 The phosphorylated PIP3 recruits Akt to PDK1 which then phosphorylates Akt (serine-tyrosine kinase) and dephosphorylates PIP3 to PIP2 to recycle this phosphate carrier. Phosphorylation of GTPase activating protein (GAP) is initiated utilizing AS160 (Akt substrate 160 kDa) which controls vesicle trafficking with by phosphorylating a Rab6a GTPase activating Protein (GAP)2. AMPK is an AMP dependent protein kinase and is an essential kinase utilized in many metabolic pathways to signal the need of immediate ATP production and has previously been targeted in pharmaceutical investigations for DMI treatment. In the case of glucose, AMPK demotes glycogen synthesis and deactivates fatty acid oxidation which is catabolic and uses energy. AMPK is naturally stimulated by heightened concentrations of AMP, the depleted form of ATP, indicating that immediate energy is needed and to cease energy expenditure, which initiates the breakdown of glucose to produce ATP.

The mechanism of GAP activity on TUG-UBL1 and activation of USP25m protease is thought to be by PIST (syntaxin-6-interacting protein) which is yet to be confirmed as an effector of GAP proposed to stimulate TUG-UBL1 cleavage. Intact TUG-UBL1 links GSVs (glucose storage vesicles) to the GSV by binding GLUT4 and IRAP (Insulin responsive aminopeptidase) through its N-terminus and Golgin-160 and other matrix proteins by its C-terminus region. TUG-UBL1 cleavage by USP25m protease separates these N and C termini and allows for TUG-UBL1 to guide GLUT4 from GSVs to the cellular membrane. This movement allows for glucose uptake into the intracellular matrix for use in glycolysis as seen in figure 10.2

The dysfunction of phosphorylation kinases throughout the insulin stimulated pathway leads to insulin resistance, especially as the cascading effect of GSV containing GLUT4 vesicles translocation to the membrane must be initiated, as this mechanism is utilized to continue glucose uptake after initial

Figure 10. TUG interaction of GLUT4 and GSV utilizing cleavage by the Usp25m protease allowing for translocation of GLUT4 to the cell surface.2
translocation has occurred. If insulin-stimulated translocation of GLUT4 is not occurring as necessary, the uptake of glucose is not possible without therapeutic intervention. Many hypotheses exist of the methods of dysfunction in this pathway, ranging from dysfunction in phosphorylation pathways\textsuperscript{13} to the USP25m protease\textsuperscript{2} with hypothesized mechanism shown in figure 10. Although, it is known that the function of this protease is essential to effectively uptake glucose from the bloodstream. This allows for a mechanism to evade diabetes type two kinase mutations by way of avoiding the insulin sensitive pathway and instead initiating the effects necessary from this pathway to uptake glucose.

**Failure of Insulin Response in Type Two Diabetes**

Additional implications of insulin resistance are of the phenomena where existing insulin resistance can cause the decrease of insulin production and secretion. The decrease in insulin production is due to residual increased blood glucose levels after insulin has been secreted from β-cells in the pancreas, which leads to cell stimulation of synthesizing more insulin. As β-cells continue to synthesize insulin in attempts to lower blood glucose levels, the cells become fatigued when no change results, and insulin production can fail as cytokine immune reaction to fatigued β-cells mount. With repeated or prolonged failures, the β-cells will be depleted to the point of no longer having the capacity to synthesize insulin which can lead to development of insulin deficient type one diabetes\textsuperscript{27} from chronic type two diabetes. Increased insulin resistance resulting in decreased secretion implies that therapies that circumvent the direct stimulation of insulin would be highly beneficial in patients with DMII. This is due to the relationship of decreased insulin secretion potentially resulting in long term insulin resistance as β-cells become fatigued. If a pharmaceutical is designed to circumvent this signaling, the decreased secretion of insulin could be prevented in patients who have not yet lost production of insulin. As the stimulation of insulin results in translocation of GLUT4 to the cell membrane, a singular event of endoproteolytic cleavage is proposed to continue the uptake of glucose after the cleavage has occurred. As glucose uptake is not stoichiometrically related to endoproteolytic cleavage, a pharmaceutical that stimulates cleavage indicates a high potential for a small amount of pharmaceutical needed to decrease blood glucose levels. This contrasts with currently prescribed medications where high concentrations are necessary to stabilize blood sugar levels, leading to large and frequent doses prescribed in DMII treatments.
TUG-UBL1 includes a UBX binding domain to GLUT4 in the N-terminal region as seen in Figure 11. This region is intact in fasting cells and is disassembled in the presence of insulin stimulation, as seen in 3T3-L1 adipocytes. It is hypothesized that TUG-UBL1 is a tether protein to GSV that allows for release and translocation of GLUT4 to the cell membranes by disrupting the bond between GLUT4 and TUG-UBL1. This binding between TUG-UBL1 and GLUT4 is found in the N-terminal region of TUG-UBL1, while the C-terminal region remains attached to the mitochondrial membrane and is necessary to retain GLUT4 intracellularly. The disruption of the N-terminal region allows for migration of GLUT4 to the cell surface along a microtubule. Endoproteolytic cleavage of TUG-UBL1 is shown to be necessary in translocation of GLUT4 transport proteins from GSV, which encapsulate a pool of GLUT4 near the TUG-UBL1 protein. Endoproteolytic cleavage separates the N-terminal region from the C-terminal region of TUG-UBL1, which allows for the release of GLUT4 from the GSV to the plasma membrane. The protease Usp25m is required to stimulate this endoproteolytic cleavage of the N and C-termini of TUG-UBL1, and is likely the protease regulated by the insulin signaling pathway to release GLUT4 from GSV to the plasma membrane. This interaction has been shown utilizing dose-dependent cleavage of TUG-UBL1 with GLUT4 upon increasing concentrations of Usp25m in 3T3-L1 adipocytes and is hypothesized to occur via the same pathway in muscle cells.

To determine the relationship of TUG-UBL1 in the protein interaction of glucose uptake by an effector molecule of GAP, PIST, transgenic mice were formed utilizing a truncated TUG-UBL1 protein that inhibits the suppression of GLUT4 in the golgi body. As knockout TUG-UBL1 mice causes embryonic lethality, UBX-Cter mice were formed where the UBX-Cter protein causes depletion of PIST and mimics the insulin stimulated state. The transgenic mice had increases in whole-body glucose turnover and overall metabolic rate. The study found that TUG-UBL1 definitively allows for movement of GLUT4 onto cell surfaces and that this action closely mimicked the symptoms of diabetes type one which indicates the necessity of TUG-UBL1 and PIST in the uptake of glucose to the cell.

The mechanism of TUG-UBL1 in response to insulin stimulation is still not fully understood, though the potential of pharmaceutical interactions in this pathway includes the possibility of inducing endoproteolytic cleavage. This cleavage would induce GLUT4
translocation possibly by activating PIST to act on the TUG-UBL1 protein. It is necessary to study both interactions to find the most promising pharmaceutical mechanism to promote GLUT4 translocation without necessitating the addition of insulin. Additionally, as PIST could activate Usp25m though the mechanism of this activation has not been determined, the interactions of PIST and Usp25m will be studied to further understand the most essential target of pharmaceuticals. This target will circumvent the insulin signaling pathway with the least off target effects as possible. Since TUG-UBL1 is specific to the GLUT4 translocation pathway, this protein is a prime target for highly specific pharmaceutical action and is anticipated to have little to no off-target activity when a pharmacophore is designed specifically for this function as seen in several approved rational design pharmaceuticals on the market.
Proposal

It is imperative to bring a therapy to market that circumvents the insulin signaling cascade for resistant patients by stimulating a specific mechanism in the insulin response pathway to initiate glucose uptake independent of cellular insulin response. It has been shown that TUG plays a central role in the insulin signaling pathway although the mechanism is not fully understood. TUG cleavage is dependent on a phosphorylation pathway where Usp25m interacts directly with TUG to cleave the protein and translocate GLUT4 for glucose uptake. TUG-UBL1 is an essential protein, as cells with disabled TUG action do not uptake glucose upon insulin stimulation. To allow such a target to exist, the mechanism must be fully elucidated for specific targeting of effector molecules in a novel pathway.

To translocate GLUT4 storage vesicles to the cell surface, TUG must be acetylated to form TUG ubiquitin-like modifier with N and C termini separated. To initiate this conformational change, insulin in the bloodstream binds to the insulin receptor subunit resulting in a phosphorylation cascade to release syntaxin-6-interacting protein (PIST) from the GAP regulator protein. PIST affects TUG activation through the USP25m protease, as data supports Usp25m association with unstimulated TUG-bound GSVs, and insulin stimulates the acute mobilization of Usp25m from the TUG C-terminus. Upregulation of Usp25m has been shown to cleave TUG with visible 42kDa and 54kDa products of the C-terminal region and 130kDa from the N-terminal region. PIST has been shown to bind to TUG, though the effect exhibited to GLUT4 translocation requires further study.

The investigation of how PIST effects the endoproteolytic cleavage of TUG by activating or deactivating Usp25m is the next step to further understanding the role of PIST mediation of glucose uptake. This increased understanding in the insulin sensitive pathway allows for a fundamental shift in diabetes related treatment discovery as a potential therapy of this sort would be the first of its kind to allow for insulin independent treatment to decrease blood glucose levels while allowing cells to utilize glucose for energy production.

TUG cleavage will be monitored utilizing MALDI/MS (Matrix Assisted Laser Desorption/Ionization mass spectroscopy) which is a key technique in mass spectroscopy related proteomics due to its extreme sensitivity, ease of application and tolerance to contamination. MALDI/MS is another form of mass Figure 12. MALDI/MS peaks anticipated in bound and non-bound products. Black represents uncleaved products of unbound TUG-UBL1 (30kDa), unbound Usp25m (130kDa), and bound TUG-UBL1-Usp25m (160kDa). Red represents cleavage products at 42kDa, 54kDa, and 140kDa.
spectroscopy which ionizes compounds into charged molecules which mass/charge ratio is read in a concentration dependent peak size. In MALDI/MS, peptides are separated into charged molecules in a “soft ionization” method where ion formation does not significantly impact sample integrity and is highly applicable for large polypeptide detection. An expected readout will be of approximately the full polypeptide size due to this soft ionization methodology. MALDI/MS is useful in purified proteins or intact cells where the size of protein products and concentration are accurately monitored. This method is highly sensitive to protein confirmation and degradation changes which is highly useful in the screening of protease effect on TUG cleavage due to the size and concentration of cleavage products as analyzed by MS shown in figure 12.

First, the binding ratio of TUG and Usp25m protease will be determined and observed for binding and cleavage affects using MALDI/MS, then the effect of PIST on cleavage will be analyzed with MALDI/MS while varying concentration of PIST and time of addition. This will elucidate the mechanism by which TUG cleavage is controlled to allow for further pursuit into pharmacological target proteins.

**Aim 1: Determination of Usp25m Concentration Necessary to Bind TUG-UBL1.**

Currently, it is known that USP25m binds to unstimulated TUG protein and the dissociation of this protease is related to the cleavage of TUG into its N-terminal and C-terminal products. This phase will determine the effects of USP25m concentration on TUG cleavage to find the necessary cellular concentration of protease to initiate cleavage. This cleavage will be monitored utilizing MALDI/MS as previously discussed.

Expected results of un-cleaved product will show bound TUG-Usp25m at 140kDa with 100% integration area and no peak area found in 42, 54, and 140kDa from cleavage products as seen in figure 12. When Usp25m effectively initiates cleavage, peaks are anticipated only at 42, 54, and 140 kDa with little to no area at 160kDa showing only cleavage products without the full-length TUG-Usp25m complex. Additionally a SDS-PAGE gel will be run on TUG protein product after treatment with varying concentrations of protease USP25m to determine if cleavage products of 42 and 54 kDa from the C-terminus and 140kDa from the USP25m bound N-terminus are present in relative abundance to full length TUG protein of 60kDa and free USP25m of 130kDa. MALDI/MS will provide sensitive quantitative analysis of USP25m concentration needed to initiate TUG-UBL1 cleavage as shown in Figure 9 while SDS-PAGE will provide a reliable and qualitative reference to MALDI/MS for further confirmation of results. Optimal molar ratio of Usp25m will determine the least unbound Usp25m by monitoring of 130kDa peak through trials of altered Usp25m concentration added and allows for further study of the TUG cleavage mechanism without free Usp25m confounding results.

The cleavage of TUG-UBL1 dependent only on USP25m will further determine the effect of this protease without external stimulation in fetal bovine serum which mimics basal cellular activity and is expected to remain in un-cleaved form when bound to only Usp25m. When insulin
stimulation is not present, USP25m surrounds TUG although a baseline activity of protease effect on TUG confirmation is needed to determine further interactions of USP25m and TUG. This is necessary to monitor the effects of this protease for diabetes related treatments, as well as a more in-depth analysis of the bio-functionality of this process.

Experimental techniques will mimic the scheme shown in figure 13. TUG-UBL1 will be obtained from 3T3-L1 adipose cells transfected by retroviruses to induce the expression of TUG in plasmids created by the Bogan lab which will be grown in E. coli to utilize regulation from lac-operon induced production of TUG with IPTG prior to purification utilizing a Q-sephadex anion exchange column as done by Singh et. al. to remove cell debris and non-target proteases present in the E. coli vehicle. Separation will be verified utilizing SDS-PAGE gel as TUG protein from this plasmid will be 60kDa and Ovalbumin and Myoglobin can be used as markers with relative sizes of 44.5kDa and 17kDa respectively.

USP25m will be obtained utilizing procedures and plasmids from the Denuc lab with purification of USP25m protease performed utilizing a Ni\textsuperscript{2+} pull down column to remove residual proteins from E. coli growth for use in TUG-Usp25m binding molar ratio determination. Utilizing a method developed by Tucher et. al., the purified TUG protein will be added increasing concentrations of USP25m in fetal bovine serum to closely mimic cellular conditions while utilizing in vitro control as done by Habtemichael et. al. and allowed to react prior to the addition of sodium carbonate to irreversibly inactivate protease activity. Samples will then be loaded into 16% SDS-PAGE gel with myoglobin, myosin, and ovalbumin markers and relative isoform concentrations validated with MALDI/MS, utilizing analysis methods devised by Parker et. al. without chromatography monitoring of protease activity due to the lack of antibody affinity beads specific for the GLUT4 binding location of TUG. Increasing Usp25m concentration will elucidate the necessary molar ratio to ensure the minimum possible amount of free Usp25m for use in effector activity studies. It is desired to have the highest concentration of bound TUG-Usp25m with minimal free TUG or free Usp25m to best understand the mechanistic effect in future investigations.

The binding of USP25m in increasing concentration will be determined by the concentration of free un-cleaved TUG protein at 30kDa, bound USP25m TUG protein at 160kDa, and monitoring of cleavage side products at 42, 54, and 130kDa which are not expected in TUG-Usp25m bound molecules without effectors added. If the reaction runs as expected the binding of USP25m will...
not induce cleavage regardless of added concentration as an affecter necessity is anticipated to separate TUG-USP25m and induce proteolytic cleavage of the TUG protein resulting in N-terminal and C-terminal products. The molar ratio concentration of USP25m which best binds TUG protein will be utilized in following experimentation to determine PIST affect in proteolytic cleavage in bound TUG-USP25m.

Aim 2: Investigation of PIST Activity on Usp25m to Regulate TUG Cleavage.

To determine the effect of PIST on the TUG-USP25m complex, PIST will be grown and separated following the scheme shown in figure 14 utilizing a method developed by Wente et al. with transfection of pAS2 vector containing PIST plasmid transfected in HEK293 cells grown on poly-d-lysin-coated glass covered slips. Cells will then be lysed in assay-lysis buffer prior to centrifugation. Further purification will be performed utilizing a Glutathione-affinity column pull-down assay utilizing His6-tag fusion protein to bind PIST. Purification of PIST proteins will be monitored by SDS-PAGE with expected size of 28kDa and concentration determined by MALDI/MS with experimental method utilized as previously described. If purification of PIST is not achieved utilizing this method, pre-purified protein ab93743 will be purchased through abcam, though this product may not be compatible for MALDI/MS analysis due to its purity and higher stability than proteins recently made by the vehicle. If proteases are degraded while trials are in place, the experiment will need alteration to match cellular conditions more concisely. In an event that MALDI/MS is ineffective at monitoring cleavage products, it may be necessary to determine mechanistic abilities although fluorescent tagging will likely be necessary to monitor the cleavage products which reduces quantitative ability to monitor PIST affect.

The determined most effective molar ratio binding concentration of USP25m to 50mM TUG will be utilized to further investigate the effect of PIST on the TUG cleavage product. Two variables of PIST concentration and order of PIST combination will be analyzed, with increasing concentrations added to pre-described sample combinations. PIST will be added to 50mM TUG bound USP2m with optimal molar ratio in fetal bovine serum as discussed previously and analyzed in 16% SDS-PAGE to

Figure 14. Experimental scheme of techniques used to determine order of addition of PIST to Usp25m and TUG to determine cleavage effects.
determine cleavage products. The PIST added to TUG-USP25m complex will be compared to a subsequent assay with USP25m combined with increasing concentrations of PIST prior to addition to TUG to form any PIST-USP25m complex prior to TUG protein interaction. The comparison of these assays will determine if PIST binds to USP25m prior to TUG-USP25m complex formation or if PIST initiates the separation of an already bound TUG-USP25m complex. The impacts of this order of binding will bring understanding of the interaction of PIST and Usp25m, as it is possible for Usp25m binding to TUG prevented by PIST which would allow for cleavage over time, or for PIST to remove Usp25m from TUG inducing cleavage. Cleavage of TUG will be analyzed by MALDI/MS for concentration determination and SDS-PAGE gel to visually monitor protease activity based on N-terminal and C-terminal products of protease cleavage as shown below in figure 15 where determinations of proteolytic ability were tested in short hairpin RNA (shRNA) modifications of TUG. If activity does not increase with concentration, protein specificity may need to be further confirmed utilizing western blot to ensure the correct protein is purified in a cellular stable conformation.

The tracking of TUG cleavage dependent on PIST addition before or after USP25m protease and PIST concentration-increasing understanding of the signaling cascade initiating TUG cleavage and allows for glucose uptake due to GLUT4 translocation. This mechanism is necessary to fully discern prior to any pharmaceutical developments for effective DMII treatment. Other known negative regulation pathways of USP25m have been described, such as in IL-17 signaling pathways with roles in inflammation. However, the regulation of Usp25m in the TUG-insulin signaling pathway mechanism regulated by GAP protein release of PIST suggests PIST is a positive regulator of USP25m cleavage. As such, this quantification of PIST effect on USP25m cleavage is necessary to elucidate the mechanism of action which PIST contributes to the insulin signaling pathway leading to glucose transport into the cell. It is known that USP25m exists in the presence of unstimulated TUG-UBL1 and is related to the cleavage of TUG to its products, although the direct effect of PIST released from the GAP mediator protein is not well understood. Greater understanding of this mechanism will be gained by altering the order of products added, allowing elucidation of PIST as a Usp25m inhibitor to prevent Usp25m-TUG binding or as an inhibitor of cleavage on the TUG-Usp25m complex. Further understanding of the TUG-UBL1 cleavage pathway in respect to USP25m protease action based on presence of PIST will enhance understanding of the pathway to initiate cellular glucose uptake. This increased understanding allows further experimental determinations to be made on possible treatment mechanisms for type two diabetic patients who have greatly reduced responses to insulin stimulation.

Figure 15. Cleavage monitoring of TUG in C-terminal region in presence of insulin with mutated TUG short hairpin RNA sequence mutations to show SDS-PAGE expected results when USP25m protease cleaves TUG protein.3

![Figure 15](image-url)
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