The Role of PFKFB3 in AMPK-Activated GLUT4 Translocation

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Abstract

Type 2 diabetes is a chronic, potentially deadly disease that impacts millions of Americans' lives. Type 2 diabetes is characterized by increased blood glucose levels caused by insulin resistance. The normal insulin signaling pathway leads to glucose uptake by GLUT4 through activation of the IRS-1-PI3K-Akt pathway, as well as the insulin-independent pathway that utilizes AMPK. Additionally, PFKFB3 may play a role in insulin signaling and glucose uptake. PFKFB3 is an enzyme that plays an important role in activating PFK-1, which is a rate-limiting enzyme in glycolysis. PFKFB3 is frequently studied for its role in cancer due to its role in cell proliferation and differentiation. However, little is known about its involvement in insulin signaling.

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Type 2 Diabetes

Diabetes is a chronic disease impacting the lives of millions of Americans. In 2021, it was the 8th leading cause of death in the United States (Ellis, 2023), and the Center for Disease Control and Prevention (CDC) estimates that about 38.4 million Americans or 11.6% of the population is currently living with diagnosed or undiagnosed diabetes. Of these 38.4 million people, 90-95% of them have type 2 diabetes. Additionally, another 97.6 million Americans over eighteen have prediabetes. Overall, 41% of Americans are currently impacted by either diabetes or prediabetes (Center for Disease Control and Prevention, 2022). Thus, the importance of understanding the causes of type 2 diabetes is critical. Developing effective treatments for this disease is contingent on understanding the cellular mechanisms through which the body develops insulin resistance.

Causes

Type 2 diabetes is characterized by insulin resistance, where the body still produces insulin, but cells do not respond as effectively to the insulin. This leads to high blood sugar and high insulin levels. Eventually, the pancreas will be unable to produce the amount of insulin needed for effective glucose uptake. There are many contributing factors to the development of type 2 diabetes. Obesity and low physical activity are linked to the development of type 2 diabetes. According to the NIH, the location of body fat also plays a role in the disease. Stomach fat is commonly associated with insulin resistance (National Institute of Diabetes and Digestive and Kidney Diseases, 2019). Insulin resistance in muscle cells can be caused by an increase in reactive oxidative species, increased levels of circulating free fatty acids, and inflammation (Shamshoum et al., 2021).

Additionally, genes and ethnicity impact the development of diabetes. People from American Indian, Alaska Native, Hispanic, and Black heritage have the highest risk for the development of diabetes. It is important to note that poverty seems to play a role in the development of type 2 diabetes. Income level is inversely correlated to the incidence of diabetes, with people living below the federal poverty level developing diabetes at almost triple the rate of people living 500% above the federal poverty level (CDC, 2022).

Long-term Complications

Type 2 diabetes, if left untreated, can lead to many serious or fatal complications. Diabetes can lead to macroangiopathy, which can cause chronic hypertension and lead to strokes and coronary artery disease. Additionally, diabetes can lead to vision loss and blindness, renal problems, and muscle atrophy. However, the most serious complications are those which impact the cardiovascular system. Most of the deaths from diabetes involve cardiovascular complications, including coronary disease, heart failure, and hypertension. These possible complications make type 2 diabetes a very dangerous disease. It can lead to a significant decrease of quality of life (Farmaki et al., 2020).

Disease Management

Type 2 diabetes is a chronic illness with no cure. However, there are many ways that people diagnosed with diabetes can improve their prognosis and quality of life while living with the disease. It is important for people to make lifestyle changes that help them manage their high blood sugar. People with diabetes should work to eat less calories and have smaller portions of food throughout their day. They should avoid processed grains, starchy food, and foods with high sugar contents, and they should instead focus on eating foods that are high in fiber and fruits and vegetables. Additionally, as type 2 diabetes is linked to obesity, people should attempt to

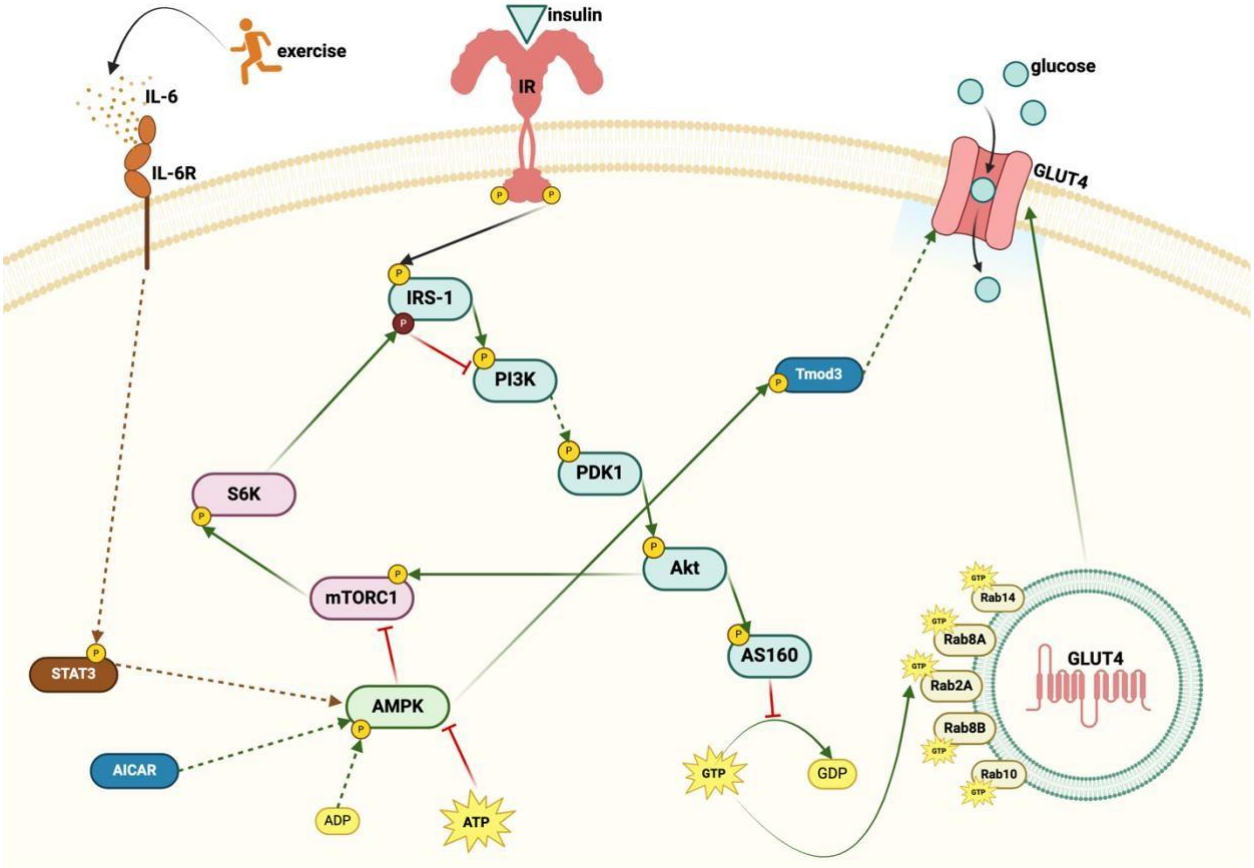
lose weight if they are overweight. Finally, people with diabetes should strive to maintain healthy physical activity habits. It is recommended that adults spend at least thirty minutes each day doing some form of cardiovascular exercise, and they should do a form of resistance exercise two or three days a week. People with diabetes should continue to monitor their blood glucose levels as they make lifestyle changes. If they continue to have high blood glucose levels, there are medications available that help the body increase its glucose uptake and lower blood sugar (Mayo Clinic, 2023).

Normal Insulin Signaling

The process of how glucose is taken up from the bloodstream into cells involves a very complex signaling pathway that is still being studied (Figure 1). Glucose uptake into adipocyte and skeletal cells is controlled by GLUT4, a protein transporter. GLUT4 is typically stored inside cells, surrounded by vesicles, making up the glucose storage compartment (GSC). These vesicles respond to insulin and help dock GLUT4 in the cell membrane when insulin is present. Once GLUT4 is docked in the cell membrane, it transports glucose from the blood into the cell. Insulin and its second messengers are crucial components to the ability of GLUT4 to translocate to the cell membrane and mediate glucose uptake (Livingstone et al., 2022).

Figure 1

Normal Insulin Signaling Mechanism



Note. This shows the insulin dependent signaling pathway through IRS-1, PI3K, and Akt that results in the translocation of GLUT4 from an intracellular storage compartment to the plasma membrane. The figure also shows the negative feedback pathway that operates through mTOR and the serine phosphorylation of IRS-1. Additionally, the figure shows the activation of AMPK through STAT3 and AICAR, leading to the phosphorylation of Tmod3. Tmod3 aids in the fusion of GLUT4 with plasma membrane. Dashed arrows indicate indirect activation. Figure created by author through BioRender.

IRS-PI3K-Akt Signaling Pathway

The insulin receptor is a tetramer, made up of two alpha subunits outside of the cell and two beta subunits inside the cell. When insulin binds to the alpha subunits extracellularly, it

causes the two intracellular beta subunits to dimerize. Additionally, insulin binding leads to the tyrosine autophosphorylation of the insulin receptor. This phosphorylation activates tyrosine kinases on the insulin receptor, and these kinases subsequently phosphorylate a tyrosine residue on the insulin receptor substrate 1 (IRS-1). The phosphorylation of IRS-1 leads to the recruitment of phosphatidylinositol 3-kinase (PI3K) and its phosphorylation by the receptor. Once PI3K is activated, it phosphorylates 3-phosphoinositide-dependent kinase 1 (PDK1), which in turn phosphorylates Akt, also known as protein kinase B (PKB). This signaling pathway initiated by insulin and propagated by a chain of phosphorylation is one of the main mechanisms through which GLUT4 translocation occurs (Zheng et al., 2021). Additionally, in some circumstances, the phosphorylation of IRS-1 activates the Raf-MEK-ERK pathway (Lee et al., 2023).

Once insulin signaling leads to the phosphorylation of Akt, Akt will phosphorylate AS160, a substrate of Akt. AS160 is an active RabGAP that is located near the GLUT4 storage vesicles (Lansey et al., 2012). RabGAPs are important vesicular transport regulators. They are GTPase activating proteins and they work by converting GTP to GDP. When GTP is bound to a Rab protein, that vesicle is activated, however, when GDP is bound to a Rab protein, the vesicle is inactivated. Thus, the RabGAPs have control over the activation or inactivation of Rab proteins (Gavriljuk et al., 2012). This RabGAP involved in insulin signaling is phosphorylated on the Arg^{973} residue of AS160 (Xie et al., 2016). When phosphorylated, the RabGAP becomes inhibited, allowing Rab to be in its GTP-bound state, allowing for the activation of Rab substrates. Under basal cell conditions when insulin is not present, the AS160 RabGAP is activated, meaning that Rab substrates are bound to GDP and inactivated (Lansey et al., 2012). The vesicles in the glucose storage compartment contains Rab2A, Rab8A, Rab8b, Rab14, and

Rab10. Thus, when the GAP activity of AS160 is inactivated due to insulin signaling, these Rab proteins become GTP-bound and are activated. They work to translocate GLUT4 from the intracellular trans Golgi network to the cellular membrane, allowing for glucose uptake to occur (Minea et al., 2005) Finally, AS160 is critical for the recycling of GLUT4 following glucose uptake. Xie et al. (2016) found that a GAP deficiency in AS160 leads to increased lysosome dependent GLUT4 degradation, and a cellular deficiency of GLUT4 transporters.

mTOR

IRS-1 is a critical mediator in insulin signaling: depending on whether tyrosine residues or serine residues are phosphorylated, IRS-1 will either be activated or degraded. The IRS-1PI3K-Akt pathway leads to activation of mTOR. mTOR controls two complexes: mTORC1 and mTORC2. mTORC1 (mammalian target of rapamycin complex I) is associated with the regulation of cell growth and proliferation, and it is a serine/threonine kinase. mTORC1 is composed of several components, including mTOR, raptor, mLST8, and PRAS40. PRAS40 functions as a negative inhibitor of mTORC1, and it is believed that raptor works to assemble the mTORC1 complex. The mTOR subunit of mTORC1 contains the kinase region. mTOR can be inhibited by the binding of rapamycin to the FRB domain in mTOR. mTOR promotes cell growth by activating eIF-binding protein (4E-BP1) (Yip et al., 2010). Additionally, mTOR activates S6K proteins, which are serine kinases that phosphorylate the serine residues on IRS-1. This serine phosphorylation leads to the degradation of IRS-1, which prohibits it from further activating its downstream signaling effectors. This creates a negative feedback loop, so activation of the IRS-1-PI3K-Akt pathway will ultimately shut itself off, preventing constant activation of Akt (Zheng et al., 2021).

AMPK

Insulin signaling through the IRS-1-PI3K-Akt pathway promotes GLUT4 translocation in an insulin-dependent mechanism. However, glucose uptake can be stimulated in an insulinindependent manner through the activation of adenosine monophosphate-activated protein kinase (AMPK). AMPK is a regulator of energy. It is a heterotrimeric protein made up of alpha, beta, and gamma domains. The alpha domain contains kinase activity, while the beta domain is regulatory. AMP, ADP, and ATP bind competitively to the gamma domain of AMPK, which leads to the ability of AMPK to sense the energy levels in the cell. When the body is in a fasted state, the AMP:ATP ratio increases. Under this state, AMP stabilizes and activates AMPK by protecting the AMPK kinase activation loop from phosphatases. AMPK is activated by the phosphorylation of the Thr^{172} residue in the activation loop. The maintenance of the phosphorylation of AMPK allows it to remain active. Additionally, AMP increases the interaction of AMPK with LKB1. LKB1 is an upstream kinase that phosphorylates the activation loop of AMPK. When the AMP:ATP ratio decreases, indicating that the body has progressed to a fed state, ATP inhibits AMPK. ATP acts as a competitive inhibitor of AMP, binding to AMPK in the place of AMP. Because ATP is bound instead of AMP, the activation loop of AMPK is no longer protected, and phosphatases can remove the phosphate from AMPK. This leads to the deactivation of AMPK. Thus, AMPK uses the AMP:ATP ratio to sense and regulate the energy state of the cell (Yan et al., 2021).

As an energy sensor, AMPK is affected by exercise. When the body undergoes exercise, it activates AMPK through contraction signaling. Exercise induces the release of the cytokine IL6. IL-6 operates through two signaling pathways: the proinflammatory trans signaling pathway involving soluble IL-6 receptors and the anti-inflammatory classical signaling pathway involving

membrane-bound IL-6 receptors. In skeletal muscle, exercise-induced classic IL-6 signaling leads to the phosphorylation of STAT3, which activates AMPK. Thus, AMPK is crucial in understanding why exercise leads to increased GLUT4 translocation and glucose uptake (Marko et al., 2020).

The specific mechanisms through which AMPK leads to increased glucose uptake and GLUT4 translocation are not fully understood. One way that AMPK may increase GLUT4 translocation is through the phosphorylation of TBC domain family, member 1 (TBC1D1). TBC1D1 is involved in the regulation of GLUT4 translocation. Additionally, AMPK may be able to increase the expression level of GLUT4 by increasing its transcription. AMPK can phosphorylate histone acetyltransferases, class II histone deacetylases, and DNA methyltransferases, which ultimately leads to increased transcription of GLUT4 (Herman, et al., 2022).

A study done by Shrestha et al. (2021) investigated the role of tropomodulin 3 (Tmod3) in AMPK-activated GLUT4 translocation. Tmod3 is an actin filament capping protein, and it has been known to play a role in insulin dependent GLUT4 translocation, as it helps GLUT4 bind to the plasma membrane. During insulin signaling, Tmod3 is phosphorylated at the Ser^{71} residue, which leads to F-actin remodeling. This remodeling is what allows GLUT4 to fuse with the membrane. The study sought to determine if the AMPK-dependent, insulin-independent signaling pathway involved Tmod3. The study found that when AMPK was activated by its upstream activator, AICAR, GLUT4 was found to have translocated to the plasma membrane, allowing for glucose uptake. However, under the same conditions with a Tmod3 knockdown, GLUT4 translocation into the plasma membrane decreased. Furthermore, the study found that GLUT4 movement to the plasma membrane was unaffected by the Tmod3 knockdown, however,

the fusion of GLUT4 with the membrane was reduced when Tmod3 was knocked down. The study by Shrestha et al. (2021) also determined that AMPK phosphorylated Tmod3 at the Ser^{25} residue, as compared to the Ser^{71} residue in insulin-dependent signaling. This phosphorylation was crucial to GLUT4 fusion with the plasma membrane, as cells that contained a mutated Tmod3 that prevented phosphorylation of Ser^{25} prevented GLUT4 from being inserted into the plasma membrane. Additionally, it was found that the phosphorylation of Ser^{25} on Tmod3 led to F-actin remodeling that allowed GLUT4 to fuse with the plasma membrane. This study indicated that AMPK-induced GLUT4 translocation was mediated by the phosphorylation of Tmod3, in a similar mechanism to the phosphorylation of Tmod3 through the insulindependent signaling pathway (Shrestha et al., 2021).

Insulin Signaling in Type 2 Diabetes

The mechanism through which insulin leads to glucose uptake is very complicated, and thus there are many aspects that could be impacted by insulin resistance and lead to diabetes. One of the main causes of insulin resistance is problems with the GLUT4 protein. People with type 2 diabetes show decreased GLUT4 plasma membrane levels, indicating problems with GLUT4 translocation. One hypothesis for this is that there are problems with GLUT4 trafficking inside the cell that lead to GLUT4 being contained in dense vesicle compartments that are less responsive to insulin signaling than the normal GSCs. A study by Livingstone et al. (2022) investigated the specifics of this. They found that people with type 2 diabetes had significantly lower intracellular GLUT4 protein levels in skeletal muscle when compared to people without diabetes. Additionally, they discovered a decrease in Syntaxin16 and Sortilin levels in people with diabetes. Syntaxin16 is an important protein that helps GLUT4 fuse with plasma membrane following its translocation. Sortilin helps form the insulin responsive vesicles (IRVs) that make

up the GSCs that store GLUT4. Low sortilin levels are linked to increased GLUT4 degradation and the inability for GLUT4 to be packaged into GSCs. Thus, the decreased GLUT4 translocation found in people with insulin resistance appeared to be partially caused by decreased levels of sorting proteins inside the cell.

Additionally, a study conducted by Xie et al. (2016) found surprising information on the role of AS160 in preventing GLUT4 degradation. It was assumed that deleting AS160 or inhibiting its RabGAP activity would promote GLUT4 translocation and lead to increased glucose uptake. However, knocking out AS160 led to hyperglycemia and increased insulin resistance. It was determined that AS160 played an important role in retaining intracellular levels of GLUT4, and a loss of AS160 led to excessive degradation of GLUT4.

In addition to diabetes creating problems with GLUT4 retention, storage, and trafficking, the insulin signaling pathway is also impacted by diabetes. The bodies of type two diabetic people are in a state of hyperglycemia and hyperinsulinemia. Their cells are unable to take up glucose from the blood, leading to high blood glucose levels, and their cells are desensitized to insulin, leading the body to release excessive amounts of insulin into the bloodstream. It is known that the serine phosphorylation of IRS-1 leads to its degradation and decreased association with PDK1, disrupting the normal pathway of insulin signaling. IRS-1 can be phosphorylated on its $Ser^{636/639}$ residue by protein kinase C, c-Jun N-terminal kinase, glycogen synthase kinase 3, inhibitory kappa B kinase, mTOR, and p70S6K. Shamshoum et al. (2021) created a hyperglycemic and hyperinsulinemic condition in a rat muscle cell line to mimic the conditions found in diabetic people, and they investigated the different effects of that condition on the IRS-1-PI3K-Akt signaling pathway. Their results indicated that this state led to an increase in the serine phosphorylation of IRS-1 coupled with a decrease in tyrosine

phosphorylation of IRS-1. Additionally, they found significant increases in the phosphorylation of mTOR and p70S6K. Thus, the researchers concluded that decreased glucose uptake in type 2 diabetes was partially caused by overactivation of the negative feedback loop of the IRS-PI3KAkt signaling pathway. Even though insulin was still binding to the insulin receptor, IRS-1 was being rapidly degraded by serine phosphorylation through the overactivation of mTOR and other serine kinases. Thus, the insulin signal had no chance to initiate the translocation of GLUT4 to the plasma membrane (Shamshoum et al., 2021).

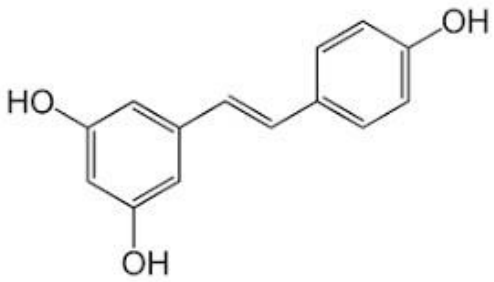
Current Therapeutic Targets

Type 2 diabetes does not have a permanent cure, but there are currently several medicines that are used to help increase glucose uptake. Additionally, researchers are continuing to work on developing new treatment options. Most of these treatments target AMPK in order to increase glucose uptake. AMPK is critically involved in metabolism and insulin sensitivity. Currently, metformin, troglitazone, and AICAR are drugs used to treat type 2 diabetes. Natural polyphenols, such as resveratrol and curcumin, have been found to activate AMPK and help aid in glucose uptake. Osthole, an extract from Chinese herbs has been studied for its effects on AMPK and GLUT4 expression. Two components of osthole, OHC-4p and OHC-2m, were found to increase AMPK and ACC phosphorylation, as well as increase p38 MAPK phosphorylation. The result was increased GLUT4 expression in the cellular membrane, indicating that osthole components could be promising therapeutic agents for diabetes (Lee et al., 2015). Figure 2 shows the chemical structures of some of the therapeutic agents.

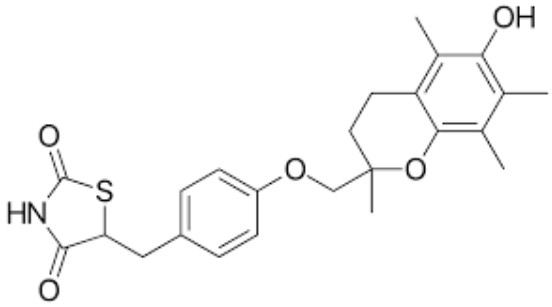
Figure 2

Chemical Structures of Therapeutic Agents

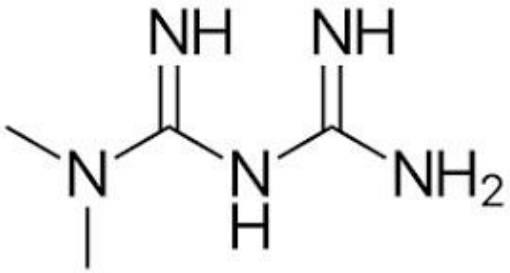
A.



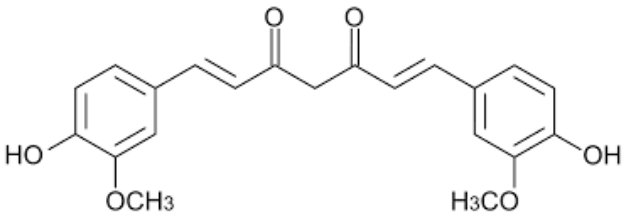
C.



B.



D.



Note. A. 3,5,4'-trihydroxy-trans-stilbene, the chemical structure of resveratrol. B. 1,1dimethylbiguanide hydrochloride, the chemical structure of metformin. C. The chemical structure of troglitazone. D. Diferuloyl methane, the chemical structure of curcumin. Structural images from Wikipedia.

Additionally, resveratrol, a polyphenol found in grapes and red wine, demonstrated promising effects in glucose uptake. Overall, resveratrol restored GLUT4 translocation and increased glucose uptake in high insulin cells. It is believed that resveratrol can increase AMPK activity. Cells treated with resveratrol showed decreased phosphorylation of mTOR and p70S6K, as well as decreased serine phosphorylation of IRS-1. These decreases in phosphorylation could be linked to resveratrol activating AMPK. AMPK inhibits mTOR, which activates p70S6K and

promotes the serine phosphorylation of IRS-1. The inhibition of the serine phosphorylation of IRS-1 prevents its degradation and promotes the PI3K-Akt signaling pathway that leads to GLUT4 translocation (Vlavcheski et al., 2020). Rosemary extract, another polyphenol, has been found to have similar effects as resveratrol on AMPK, mTOR, P70S6K, IRS-1 phosphorylation, and glucose uptake (Shamshoum et al., 2021).

Finally, *Spatholobus suberectus* (*Ss*) has been studied as a potential therapy for diabetes and hyperglycemia. *Ss* is a plant containing polyphenols, quinones, steroids, fatty acids, and procyanidins. It was found that hyperglycemic cells treated with *Ss* led to increased GLUT4 activation and increased glucose uptake through both the PI3K-Akt signaling pathway and the AMPK pathway. *Ss* increased the phosphorylation of AMPK and Akt. Additionally, *Ss* led to increases in IRS-1 and GLUT4 mRNA expression. These results demonstrated that AMPK is a central target in treatments for type 2 diabetes (Zhao et al., 2017). Discovering new ways to activate AMPK appears to be a promising approach in developing new therapeutics for type 2 diabetes.

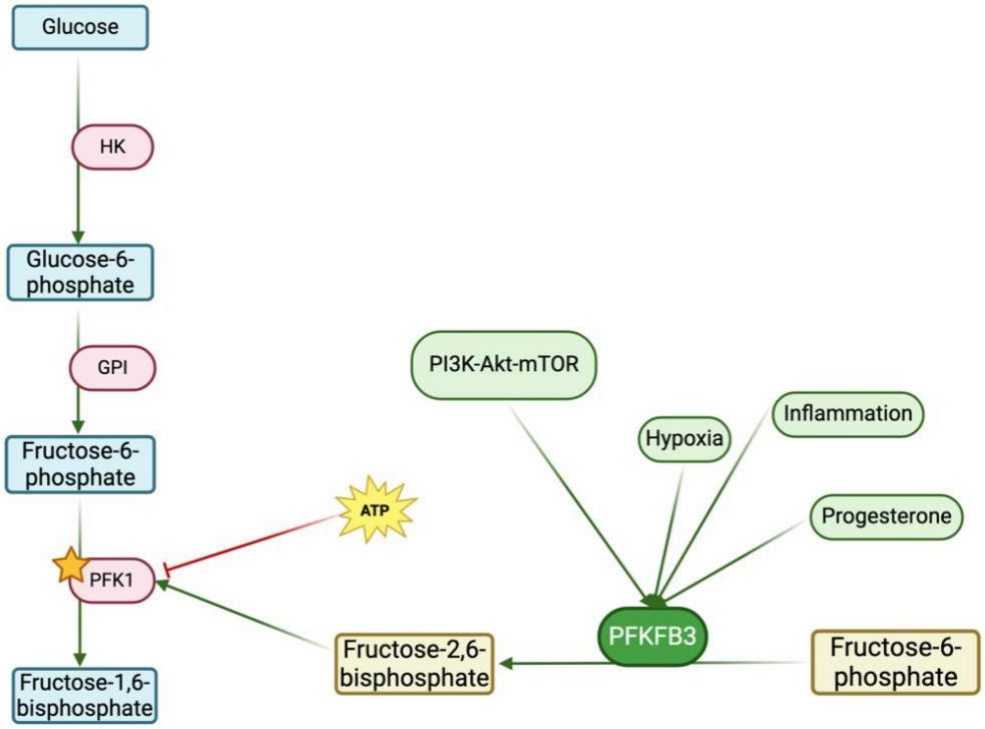
Overview of PFKFB3

Once glucose is brought into the cell via GLUT4 transporters, it can be metabolized through two separate pathways. Glycolysis degrades glucose and results in the synthesis of ATP, whereas the pentose phosphate pathway results in the formation of NADPH (Griesel et al., 2021). Glycolysis is the main pathway through which glucose is metabolized, and it can occur in both aerobic and anaerobic conditions (Figure 3). Glycolysis begins in the cytoplasm of the cell, where glucose is converted to glucose-6-phosphate by hexokinase. Glucose-6-phosphate is then converted into fructose-6-phosphate by phosphoglucose isomerase. Fructose-6-phosphate is phosphorylated by phosphofructokinase-1 (PFK1) to become fructose-1,6-bisphosphate. This

step is a rate limiting step of the metabolism of glucose, meaning that PFK-1 can regulate the speed in which glucose is broken down. However, PFK-1 is allosterically activated by fructose-2,6-bisphosphate, which is made from the glycolytic intermediate, fructose-6-phosphate. Fructose-6-phosphate is converted to fructose-2,6-bisphosphate by phosphofructokinase-2 (PFK2; Lu et al., 2017).

Figure 3

The Role of PFKFB3 in Glycolysis



Note. This shows the first three steps of glycolysis, including the rate limiting step controlled by PFK1. PFK1 is inhibited by ATP and allosterically activated by fructose-2,6-bisphosphate. PFKFB3 regulates the synthesis and degradation of fructose-2,6-bisphosphate. PFKFB3 is activated by hypoxia, inflammation, progesterone, and the PI3K-Akt-mTOR signaling pathway. Figure created by author through BioRender.

PFK-2 is a part of the PFKFB family of enzymes that is coded for by the *Pfkfb* gene. There are four *Pfkfb* genes that code for four different isoforms of the PFKFB enzyme. Of specific interest is 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) (Duran et al., 2009). PFKFB3 is considered a bifunctional enzyme: it is involved in both synthesis and degradation of fructose-2,6-bisphosphate. The N-terminus of the enzyme contains the 6phosphofructo-2-kinase, which is responsible for the synthesis of fructose-2,6-bisphosphate from fructose-6-phosphate. The C-terminus contains fructose-2,6-bisphosphatase, which is responsible for breaking fructose-2,6-phosphate back down to fructose-6-phosphate. The different isoforms of the PFKFB enzymes contain different ratios of kinase to phosphatase activity. PFKFB3 contains the highest kinase activity of the four enzymes, with its kinase function being significantly stronger than its phosphatase activity. Thus, PFKFB3 will generally favor the synthesis of fructose-2,6-bisphosphate, and thus it promotes glycolysis. The *Pfkfb3* gene is located on chromosome 10p15.1 (Lu et al., 2017). PFKFB3 is degraded through the ubiquitinproteosome proteolytic pathway. Additionally, the PFKFB3 is activated by the presence of progesterone, inflammation, and hypoxia (Duran et al., 2009). PFKFB3 is an important regulator of glycolysis because it allows for glycolysis to remain activated even in the presence of ATP. ATP normally inhibits the action of PFK-1, which ultimately slows down all of the glycolytic processes. However, when PFKFB3 is active it is able to synthesize fructose-2,6-bisphosphate, which will allosterically activate PFK1, even in the presence of ATP (Lu et al., 2017).

PFKFB3 and Cancer

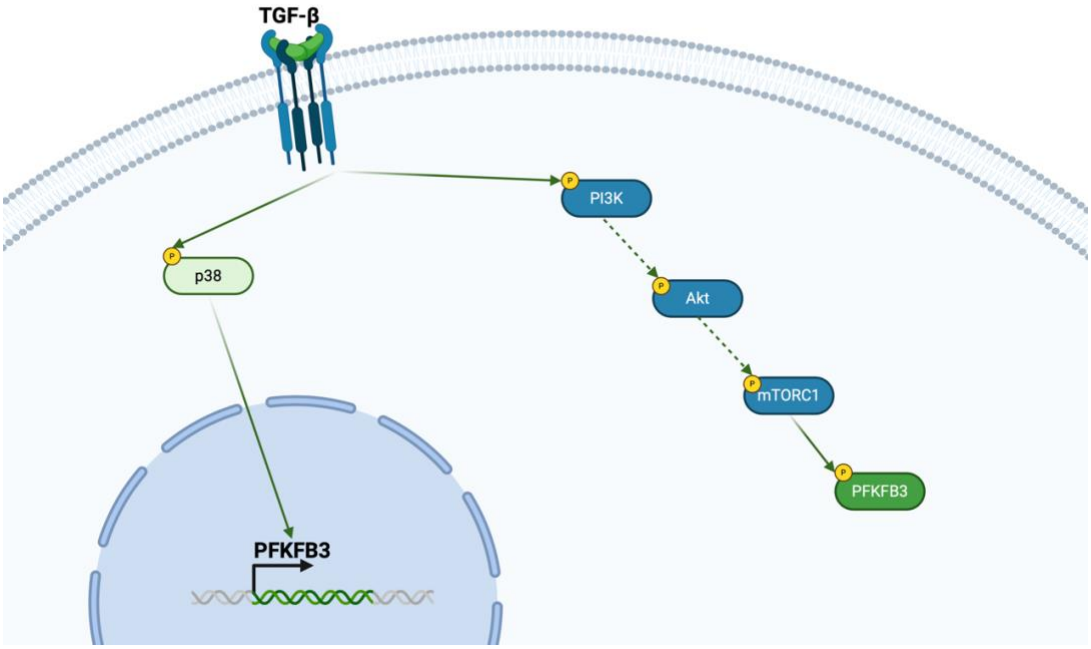
PFKFB3 is a complicated enzyme, and many of its specific functions are still not fully understood. However, as PFKFB3 is associated with glycolysis, which is necessary for the

growth and proliferation of cells, it is frequently studied for its impact in cancer. Cancerous cells proliferate uncontrollably, and many studies have found that PFKFB3 may be involved in excessive cell growth and division. PFKFB3 is found in many types of cells throughout the body, including hepatocytes, macrophages, and epithelial cells. The transcription of PFKFB3 mRNA can be increased by the transcription factors HIF1a and NF-κB. Additionally, PFKFB3 expression is activated by p38 MAPK. This is supported through research findings that showed that the inhibition of p38 MAPK inhibited PFKFB3 expression (Mager et al., 2023).

PFKFB3 protein levels have been shown to be elevated in breast cancer, gastric cancer, and bladder cancer (Wang et al., 2018). Many studies have been done in order to determine the specific mechanisms through which PFKFB3 is involved with the proliferation of cancerous cells. Studies in human glioblastoma cells investigated the interaction between the transforming growth factor-β (TGF-β) signaling pathway and the PFKFB3 signaling pathway (Figure 4).

Figure 4

Activation of PFKFB3 Through PI3K-Akt-mTORC1



Note. This figure shows how TGF-β leads to increased transcription and phosphorylation of PFKFB3. TGF-β activates the PI3K-Akt-mTORC1 pathway, which leads to the serine phosphorylation of PFKFB3. Additionally, TGF-β can activate p38, which increases the transcription of PFKFB3. Dashed lines indicate indirect activation. Figure created by author through BioRender.

TGF-β activates p38, ERK, JNK, MAPK, and PI3K; additionally, increases in glycolytic activity have been found to be linked to TGF-β. Additionally, TGF-β led to the upregulation of PFKFB3 mRNA, as well as increased phosphorylation at the Ser^{461} residue on PFKFB3. This phosphorylation activated PFKFB3, which led to increases in the amount of fructose-2,6bisphosphate. Further research on the cells was conducted to determine which specific

pathway TGF-β utilizes to increase glycolysis. TGF-β led to increased phosphorylation of both Akt and p38. Researchers then inhibited PI3K, and they noticed that PFKFB3 mRNA and proteins levels decreased, and these decreases were accompanied with a decrease in fructose-2,6-bisphoshpate, which indicated a decrease in glycolysis. Thus, it appears that TGF-β signals through the PI3KAkt pathway increase both transcription and phosphorylation of PFKFB3 (Rodriguez et al., 2017).

In most cells, Akt activation leads to the activation of mTORC1. mTORC1 is one of the two subunits of mTOR, and it controls cell growth and proliferation. Additionally, Akt can lead to the inhibition of AMPK. However, AMPK works reciprocally on Akt, and AMPK activation leads to the activation of Akt. This was demonstrated in human colorectal cells that were treated with metformin, an AMPK activator. When these cells were exposed to metformin, an increase in PFKFB3 expression was observed. This pointed to the important role of Akt in the activation of PFKFB3. AMPK was activated through metformin treatment, and an active AMPK phosphorylated and activated Akt. This Akt activation led to the activation of mTORC1, which subsequently increased PFKFB3 phosphorylation. To further test this theory, PI3K was again inhibited with LY294002, and mTOR was inhibited with rapamycin. Under these conditions, autophagy was induced. This indicated that cell growth was not encouraged due to glycolysis being slowed down. These results further pointed to the PI3K-Akt-mTORC1 pathway being critical in both the upregulated transcription and allosteric activation of PFKFB3 (Park et al., 2022).

The PI3K-Akt-mTORC1 signaling pathway is crucial in activating PFKFB3. However, further research was done to determine whether activation of PFKFB3 impacted other enzymes or signaling pathways outside of its impact on glycolysis. PFK-15 is a small molecule inhibitor

of PFKFB3, which has been known for its anti-tumor effects. Rhabdomyosarcoma (RD) cells were treated with PFK-15, and the phosphorylation levels of AMPK and ACC were observed. After 2 hours of treatment with PFK-15, AMPK phosphorylation decreased by 44%, and ACC phosphorylation decreased by 20%. After 12 hours of PFK-15 treatment, AMPK phosphorylation had decreased 88% from normal levels, and ACC phosphorylation had decreased by 61% from normal levels. These results were surprising, because it was known that AMPK activation led to increased expression of PFKFB3. However, this study suggested that there may be some instances where PFKFB3 is an upstream regulator of AMPK. This study was repeated with 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), which is a second type of PFKFB3 inhibitor, and the results were very similar. The downregulation of AMPK was reversed when these cells were treated with AICAR. The specific mechanism through which PFKFB3 would activate AMPK was not found, but this finding could prove to be very significant in the medical community (Wang et al., 2018).

PFKFB3 and Diabetes

The mechanism through which PFKFB3 is activated has been identified in cancerous cells, and the implications of PFKFB3's role in the proliferation of cancer cells has been explored in great detail. However, as PFKFB3 is an important regulator of glucose metabolism, it seems reasonable to explore whether this enzyme plays a role in the development or progression of type 2 diabetes. Cells experiencing insulin resistance are very different from cancerous cells, so it cannot be assumed that PFKFB3 will act the same way in these cells as it did in the cancerous cells previously studied. However, tumor cells and cancerous cells sometimes experience the same conditions. For example, tumor cells, inflamed cells, and diabetic cells often experience hypoxia, which is defined as a state where there is more demand

for oxygen than there is supply. Under hypoxic conditions, an increase in glycolytic action is observed (Fuhrmann et al., 2022).

In addition to hypoxia, diabetic and cancerous cells can both experience increased levels in lipopolysaccharides (LPS). While increased LPS does not mean a cell is diabetic, diabetes can develop due to consistently elevated LPS levels. A study examined the impacts of LPS on pulmonary fibrosis. It was found that LPS-induced mTORC1 activation caused collagen synthesis through the activation of glycolysis through PFKFB3. However, when these MRC-5 cells were treated with metformin, AMPK was activated and PFKFB3 was downregulated, leading to decreased collagen synthesis and decreased fibrosis. This study demonstrated that LPS-induced cellular damage functioned through PFKFB3, and this damage was reduced with metformin, a diabetes treatment (Tang et al., 2021).

PFKFB3 Expression and Disease Progression

These results do not support the conclusion that PFKFB3 plays a role in preventing insulin resistance. These studies contradict some of the studies of PFKFB3 in cancer cells. The study conducted by Tang et al. (2021) found that AMPK activation led to the decrease in PFKFB3 activity, whereas previous studies in cancer cells found that AMPK activation increased PFKFB3 activity. However, it is important to note that the same enzymes can act differently and through different mechanisms in different types of cells. A study on type 1 diabetes investigated the role of PFKFB3 in a mouse model. Type 1 diabetes is caused by degradation of pancreatic β cells, leading to hepatomegaly and increased cell proliferation. Thus, it was not surprising that streptozotocin (STZ) mice, mice that represented a diabetic form, showed increased *Pfkfb3* gene expression, increased PFKFB3 protein levels, and increased levels of fructose-2,6-bisphosphate, as increased PFKFB3 expression and protein levels are related to increased proliferation.

Similarly to previous studies done in cancer cells, inhibiting PI3K and mTOR in these mice led to a 20% decrease in PFKFB3 protein levels. This study demonstrated that PFKFB3 was also regulated through the PI3K-mTORC1 signaling pathway in diabetic cells, similar to its regulation in cancerous cells. This study also showed that type 1 diabetes led to the overexpression of PFKFB3 and an increase in glucose metabolism (Duran et al., 2009).

A second study on PFKFB3 and diabetes echoed the previous results: overexpression of PFKFB3 in relation to diabetes led to injury. This particular study investigated diabetic neuropathy, which impacts 20-40% of patients with diabetes. Diabetic neuropathy is characterized by podocyte injury in kidney epithelial cells. High glucose conditions in MPC5 mouse podocytes led to increased activation of glycolysis, which caused podocyte injury. This study found that high glucose led to decreased podocyte cell viability, cellular apoptosis, changes in the shape and area of podocyte cells, decreased phosphorylation of AMPK, and increased phosphorylation of mTORC1. However, when PFKFB3 was knocked down, all the negative effects of high glucose conditions on podocytes were reversed. Cells regained viability and retained their normal shape. These studies suggest that PFKFB3 may exacerbate the problems of diabetes by increasing cell proliferation, which can lead to tissue injury in the liver and kidneys (Zhu et al., 2021).

PFKFB3 as a Therapeutic Target

Despite the studies that demonstrated that PFKFB3 may be upregulated during diabetes, or the expression of PFKFB3 may be linked to diabetes-induced injuries, other studies have suggested the opposite. One such instance involves insulin-like growth factor 1 (IGF-1). IGF-1 is produced from the liver, and its role is to maintain insulin sensitivity and glucose uptake. While IGF-1 is involved in glucose uptake, it only accounts for 1-2% of insulin activity. Instead, IGF-1

works in more of a regulatory role. Circulating IGF-1 interacts with insulin-like growth factor binding protein 1 (IGFBP-1). Once bound to IGFBP-1, IGF-1 can activate the PI3K-Akt-mTORC1 pathway. When IGF-1 was impaired, insulin resistance could be promoted. Additionally, low circulating IGF-1 levels have been linked to the development of type 2 diabetes. It was demonstrated that overexpressing PFKFB3 could lead to positive regulation of IGF-1 and the insulin pathway (Kasprzak, 2021).

A second study investigated the role of PFKFB3 in IGF-1 signaling. This study investigated 3T3-L1 adipocytes, as PFKFB3 is highly expressed in adipocytes. When PFKFB3 was knocked down in these adipocyte cells, GLUT4 translocation due to IGF-1 signaling was impaired. The cells experienced a 22% reduction in glucose uptake when PFKFB3 was removed. A second trial of this study used 3PO, a PFKFB3 competitive inhibitor. When the cells were treated with 3PO, GLUT4 translocation was inhibited, which led to decreased glucose uptake. When PFKFB3 was overexpressed, insulin-induced phosphorylation of Akt was increased. This study demonstrated that PFKFB3 was involved in insulin signaling (Trefely et al., 2015). Because PFKFB3 is so highly expressed in adipocytes, it makes sense to further investigate its role in adipocyte maturation. To mature, adipocytes need glycolysis to create ATP and promote cell growth. 3T3-L1 preadipocytes were studied over the course of six days to determine what enzymes were crucial in their maturation. It was found that glucose uptake reached its peak at day three. At day three, the only molecule in these cells that was upregulated was PFKFB3. PFKFB3 expression was lowered on day six, similar to how glucose uptake was lowered. This led to the development of the hypothesis that glucose uptake and glycolysis are dependent on PFKFB3. To test this theory, PFKFB3 was knocked down with siRNA. After the knockdown, there was a 23% decrease in glucose uptake on day three and reduced GLUT4 translocation by

day six. This indicated that PFKFB3 plays an important role in facilitating GLUT4 translocation and glucose uptake. Mature adipocyte cells were also investigated. PFKFB3 was found in lower levels in the fasted state, but after the animal ate, PFKFB3 levels increased. When PFKFB3 was knocked out in mature mice, adipocytes decreased in cell size and glucose tolerance. However, when PFKFB3 was overexpressed through an AP2 promoter, insulin resistance was improved, inflammation of the cells decreased, and lipid storage increased. These studies suggested that there could be a potential for PFKFB3 to be targeted by a therapeutic agent for type 2 diabetes (Griesel et al., 2021).

Future Research

There are many conflicting studies on the role of PFKFB3 in insulin signaling, GLUT4 translocation, and glucose uptake. There are some studies that indicate the expression of PFKFB3 is pathological and leads to the damage and injury that is observed in type 2 diabetes. However, there are other studies that claim that PFKFB3 is under-expressed in cells during type 2 diabetes, and that lack of expression leads to problems with GLUT4 translocation. Future research must be done to determine the true role of PFKFB3 in type 2 diabetes. However, it is possible that the enzyme has different effects in different types of cells and under different conditions. PFKFB3 overexpression could be pathological in some cells and could be beneficial in other cells at the same time.

However, as research has established that PFKFB3 is activated by the PI3K-Akt-mTOR pathway, it seems reasonable to hypothesize that PFKFB3 is under-expressed in type 2 diabetes conditions. Because insulin resistance in type 2 diabetes occurs through a breakdown of the PI3K-Akt signaling pathway, it would not be surprising to see a decrease in PFKFB3 expression. Additionally, many research articles suggest that AMPK can activate PFKFB3. However, some

studies suggest that there are times where PFKFB3 could activate AMPK. AMPK is critical in the glucose uptake process. If PFKFB3 is under-expressed due to a breakdown in the PI3K-Akt pathway, there could be a chance that AMPK could experience a decrease in activation due to the decreased levels of PFKFB3 expression. However, this connection would need to be studied further. The exact mechanism through which PFKFB3 potentially regulates AMPK has yet to be discovered. Additionally, the conditions during which PFKFB3 acts as an upstream regulator for AMPK has also yet to be discovered. Finally, much of the research done on PFKFB3 was done on cancer cells or adipocytes. However, skeletal muscle is the largest site of insulin-induced glucose uptake. Future research should investigate the role that PFKFB3 plays in GLUT4 translocation and glucose uptake in skeletal muscle.

A potential research study could attempt to determine whether PFKFB3 is a necessary intermediate in AMPK-activated GLUT4 translocation in myocytes. It would be hypothesized that when PFKFB3 is knocked out, AMPK phosphorylation will decrease, and GLUT4 plasma membrane levels will decrease. Glut4myc L6 mouse muscle cells would be purchased, grown, and differentiated. The PFKFB3 gene would be knocked out of half the cells with guide RNA (gRNA) using a WT SpCas9 protein to create a double stranded break in exon two of PFKFB3. Western blots could be used to determine AMPK phosphorylation. PFKFB3 and Knockout PFKFB3 would be analyzed using an immunofluorescence staining assay. Finally, GLUT4 plasma membrane levels would be analyzed through a GLUT4myc translocation assay. It would be expected that the wildtype PFKFB3 cells would display higher AMPK phosphorylation and higher GLUT4 plasma membrane levels compared to the PFKFB3 knockout cells.

Conclusion

Type 2 diabetes is prevalent in the United States of America, impacting millions of people each year. If left unchecked, type 2 diabetes can lead to a deterioration of health or even

death. Type 2 diabetes is characterized by a lack of insulin sensitivity in cells. Cells take up glucose from the blood through GLUT4 transporters, which are normally stored in vesicles inside the cell. However, upon insulin binding to a receptor in the cell's membrane, a signal is passed through the PI3K-Akt pathway to promote the translocation of GLUT4 from inside the cell to the plasma membrane. However, type 2 diabetes leads to insulin resistance that stems from a breakdown in the signaling pathways of the cell. Ultimately, type 2 diabetes inhibits GLUT4 translocation, which prevents glucose from being taken up from the blood into the cell. This leads to chronically elevated blood glucose levels, which can cause damage to organs throughout the body. Additionally, there is a second pathway through which GLUT4 can be translocated. This pathway involves the activation of AMPK. AMPK activation has become a common target for many therapeutic treatments for type 2 diabetes, such as metformin and AICAR.

Glucose uptake leads to glucose metabolism through glycolysis. The rate limiting step of glycolysis is regulated by PFK1, which can be activated by fructose-2,6-bisphosphate. An enzyme named PFKFB3 controls the synthesis of fructose-2,6-bisphosphate, which means that PRFKFB3 can regulate the speed at which glycolysis progresses. PFKFB3 has been targeted in cancer research as one of the enzymes that causes cancerous cell growth and uncontrolled proliferation. However, there is some research on the role of PFKFB3 in type 2 diabetes, and some of this research appears contradictory and conflicting. Some research claims that PFKFB3 is overexpressed in type 2 diabetes and promotes injury. However, others suggest that PFKFB3 is actually inhibited in type 2 diabetes, and this inhibition leads to decreased GLUT4 translocation.

These conflicting reports of the role of PFKFB3 in insulin signaling indicate that further research needs to be conducted. Research should focus on the way in which PFKFB3 can

influence AMPK activation in relation to insulin signaling and glucose uptake. Additionally, much of the research that indicates that PFKFB3 could be a therapeutic target for type 2 diabetes was conducted in adipocytes. Future research should investigate the role of PFKFB3 in skeletal muscle and liver cells. Additionally, it appears that PFKFB3 operates very differently in different cells. In considering targeting this enzyme to help increase glucose uptake in some cells, it would be important to ensure that the treatment does not lead to problems developing in other cells throughout the body. Overall, PFKFB3 has the potential to be a promising therapeutic target in type 2 diabetes, but its role in GLUT4 translocation needs to be further investigated.

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