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Improving In Vivo Adenoviral Transduction for Detailed Studies of GLUT4 and AS160 Studies in Adipocytes

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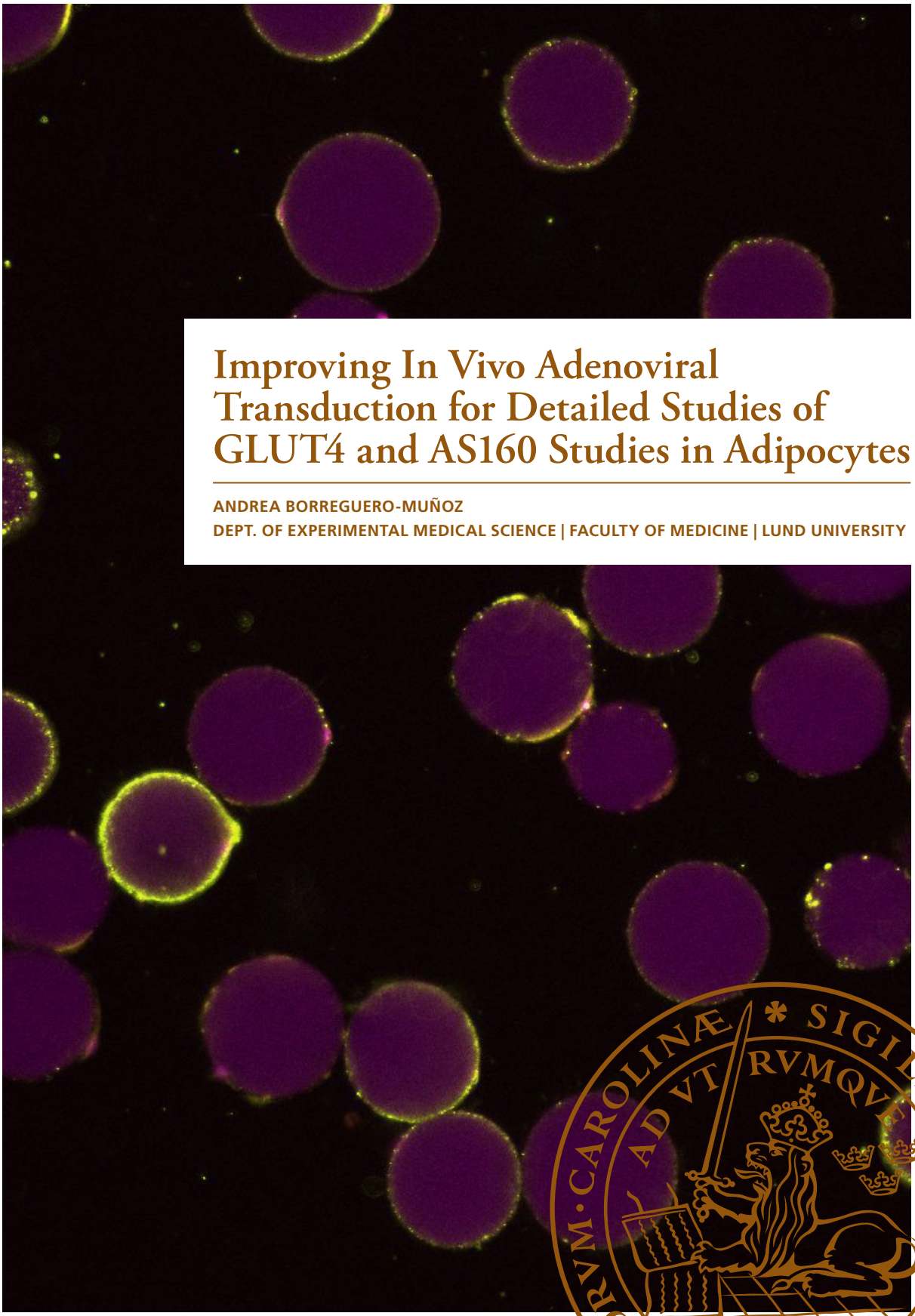

Improving In Vivo Adenoviral Transduction for Detailed Studies of GLUT4 and AS160 Studies in Adipocytes

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Abstract:

Obesity and type 2 diabetes are increasing at alarming rates worldwide, presenting significant public health challenges. A key player in these conditions is GLUT4, a protein that ~~helps~~ transports glucose into cells in response to insulin. Proper function of GLUT4 is crucial for maintaining healthy blood sugar levels and overall metabolic health.

To address this, in this thesis I have explored the molecular mechanisms influencing insulin action with a focus on GLUT4 trafficking in adipocytes. Given the limitations of traditional in vitro models, we optimized an *in vivo* adenoviral transduction method to express HA-GLUT4-GFP in perigonadal white adipose tissue. This approach allowed us to observe GLUT4 dynamics in freshly isolated adipocytes under basal and insulin-stimulated conditions using TIRF microscopy.

Using this method, we examined the role of AS160, a protein crucial for controling GLUT4 distribution. Our observations showed that in wilde type mice, insulin prompted GLUT4 to move to the cell surface, facilitating glucose uptake. However, in AS160-deficient mice, GLUT4 was already present on the cell surface without insulin stimulation. Thus, this method serves as a useful tool to accurately explore GLUT4 behavior, which could generate valuable insights into the mechanisms underlying insulin action and glucose uptake. Findings using this method could lead to better therapeutic strategies for managing and treating metabolic diseases like diabetes and obesity.

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Coverphoto: *10x magnification image of adipocytes labeled with DRAQ5 and GLUT4.*
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Table of Contents

Abstract 7
List of Accompanying Manuscripts 8
Abbreviations 9

Background 11
Obesity and type 2 Diabetes 11
White Adipose Tissue 11
Adipose tissue-signalling and functions 14
Adipocyte models and experimental approaches in GLUT4 trafficking research 20

Materials and methods 25

Overall aim and specific aims 29
General Aim 29
Specific aims 29

Results and discussion 30
In vivo adenoviral transduction of perigonadal adipose tissue to assess GLUT4 trafficking in freshly isolated adipocytes. 30
Concluding remarks 36

Popular summary 38

Acknowledgements 39

References 41

Abstract

Obesity and type 2 diabetes are increasing at alarming rates worldwide, presenting significant public health challenges. A key player in these conditions is GLUT4, a protein that helps transports glucose into cells in response to insulin. Proper function of GLUT4 is crucial for maintaining healthy blood sugar levels and overall metabolic health.

To address this, in this thesis I have explored the molecular mechanisms influencing insulin action with a focus on GLUT4 trafficking in adipocytes. Given the limitations of traditional in vitro models, we optimized an *in vivo* adenoviral transduction method to express HA-GLUT4-GFP in perigonadal white adipose tissue. This approach allowed us to observe GLUT4 dynamics in freshly isolated adipocytes under basal and insulin-stimulated conditions using TIRF microscopy.

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Abbreviations

AC	Adenylate cyclase
ACC	Acetyl-CoA carboxylase
ACLY	ATP-citrate lyase
AKT/PKB	Protein kinase B
AS160	Akt substrate of 160 kDa
AV	Adenovirus
BSA	Bovine serum albumin
cDNA	Complementary DNA
cAMP	cyclic-Adenosine monophosphate
C/EBPs	CCAAT/enhancer binging proteins
ChREBP	Carbohydrate response element-binding protein
DTT	Dithiothreitol
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FA	Fatty acid
FAS	Fatty acid synthase
GAP	GTPase-activating protein
GFP	Green fluorescent protein
GLUT	Glucose transporter
GLUT4	Glucose transporter 4
GSVs	GLUT4 storage vesicles
HA	Hemmaglutinin
HSP90	Heat shock protein 90
HSL	Hormone sensitive lipase
IL-6	Interleukin-6
IRS	Insulin receptor substrate
KO	Knock out
KRBH	Krebs-Ringer bicarbonate HEPES buffer
MSD	Mean square displacement

PBS	Phosphate-Buffered Saline
PDE3B	Phosphodiesterase 3B
PDK1	Phosphoinositide-dependent kinase-1
PKA	Protein kinase A
PPAR-γ	Proliferator-activated receptor gamma
Rab	Rat- sarcoma virus-related proteins in the brain
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SDS	Sodium dodecyl sulphate
T2D	Type 2 diabetes
TAG	Tri-acyl glycerides
TIRF	Total internal reflection fluorescence
TNFα	Tumour necrosis factor α
WAT	White adipose tissue
WT	Wild type

Background

Obesity and type 2 Diabetes

Over the past decades, obesity and overweight have become a growing public health issue in most industrialized and developing countries. Around 20% of the world population was obese in 2016 (Okunogbe et al., 2022). Obesity occurs when energy intake and consumption are imbalanced. Obesity is the main risk factor behind insulin resistance, characterized by impaired insulin response in insulin target tissues. This can cause b-cell failure and impaired insulin secretion, leading to the onset of Type 2 Diabetes (T2D) (Barazzoni et al., 2018). TD2 has steadily increased over the past decades, affecting 6.3% of the world's population according to the World Health Organization (Okunogbe et al., 2022). In obese individuals the main metabolic pathways in adipocytes are altered, causing adipocyte disfunction (Barazzoni et al., 2018). Unravelling the initial mechanisms of adipocyte dysfunction is crucial for designing effective therapies to preserve adipocyte function and combat obesity-related complications (Guilherme et al., 2008). To further study the cellular mechanisms that restrict glucose transport as adipose tissue expands, I investigate adipocyte factors controlling glucose uptake and glucose transport at a cellular level.

White Adipose Tissue

Mammalian adipose tissue is categorized into brown adipose tissue, which is involved in thermoregulation, and white adipose tissue (WAT), which primarily manages energy storage and homeostasis Given that my study focuses on WAT, the subsequent discussion will concentrate on its role in energy metabolism. When energy intake exceeds expenditure, WAT stores excess energy as triglycerides. During fasting, these triglycerides are hydrolysed and released into the bloodstream to be used as energy sources (Lafontan, 2012).

Two important proteins expressed in WAT are Hormone-Sensitive Lipase (HSL) and Glucose Transporter 4 (GLUT4). HSL is crucial for the mobilization of stored lipids by hydrolysing triglycerides into free fatty acids and glycerol during fasting. GLUT4, on the other hand, is involved in glucose uptake by adipocytes, and its

activity is regulated by insulin. Together, these factors are essential for maintaining metabolic balance, influencing lipid metabolism, and modulating insulin sensitivity (Lafontan, 2012).

In addition to its role in energy storage, WAT functions as an endocrine organ, secreting various hormones and cytokines that regulate metabolism, inflammation, and immunity (Lafontan, 2012). Hormones uniquely produced and secreted by adipocytes include leptin and adiponectin. Leptin regulates energy balance and appetite, while adiponectin enhances insulin sensitivity and exhibits anti-inflammatory properties. In contrast, cytokines such as tumour necrosis factor-alpha (TNFα), which are often secreted by inflammatory cells within WAT, contribute to inflammation and insulin resistance (Lafontan, 2012).

Humans have two types of white adipose tissue (WAT): subcutaneous, which is located beneath the skin, and visceral, which surrounds the internal organs. Visceral adipose tissue is more metabolically active compared to subcutaneous tissue and is associated with several health issues. It is linked to increased insulin resistance, particularly in the context of obesity. This form of adipose tissue is a key contributor to metabolic syndrome, a combination of conditions that includes obesity, hypertension, and dyslipidaemia, which together heighten the risk of cardiovascular diseases and type 2 diabetes (Burgermeister et al., 2003). The increased metabolic activity of visceral fat leads to elevated levels of inflammatory cytokines and free fatty acids, which impair insulin signalling and contribute to insulin (Burgermeister et al., 2003; Gupta & Gupta, 2024). Differences in insulin sensitivity between lean and obese individuals, as well as across different fat depots, affect the body's ability to store excess fat. Unlike visceral fat, subcutaneous fat is generally associated with a lower risk of type 2 diabetes and is thought to have protective effects against metabolic disturbances. It stores excess fat more effectively, without causing the same degree of metabolic dysfunction (Gupta & Gupta, 2024). Interestingly, subcutaneous fat is generally associated with a lower risk of metabolic dysfunction and stores excess fat more effectively without causing the same degree of metabolic imbalance as visceral fat. Visceral fat, on the other hand, secretes more pro-inflammatory cytokines, further worsening metabolic disturbances (Arner, 1998; Berg & Scherer, 2005).

Studies suggest that subcutaneous fat may act as a buffer, helping to sequester lipids that would otherwise accumulate in visceral fat and other organs, where they could cause more harm (Arner, 1998). In the same studies, it was shown that subcutaneous fat can mitigate the adverse effects of excess visceral fat. For example, research involving the transplantation of subcutaneous fat to visceral areas in mouse models demonstrated improvements in metabolic parameters, including enhanced insulin sensitivity and a reduced risk of metabolic syndrome (Arner, 1998).

Adipose tissue expansion

When energy intake surpasses expenditure, the body must store the excess energy. This surplus is stored as triglycerides within adipose tissue. To accommodate this increased storage demand, adipose tissue rapidly expands by increasing both the adipose cell size of existing adipocytes (hypertrophy) and cell number through recruitment and differentiation of precursors (hyperplasia) (Li et al., 2016). Excessive hypertrophy is a key characteristic of impaired adipose tissue function, and large adipocytes are known to have reduced insulin response and impaired capacity to store additional fat. Instead, excess energy accumulates in other organs (liver, muscle, and visceral adipose tissue) as ectopic fat, where it contributes to peripheral insulin resistance, a hallmark of T2D (Gustafson et al., 2015). Therefore, understanding the underlying causes of insulin resistance is crucial for developing effective treatments and preventive strategies for T2D (Colosia et al., 2013; Hruby & Hu, 2015).

Insulin-resistant individuals often have more small adipocytes, which suggests a compensatory response to impaired expansion of mature fat cells. The body tries to store excess fat by producing more small adipocytes, indicating a disruption in normal adipocyte maturation. This inability to store fat efficiently leads to fat accumulation in other organs, such as the liver and muscle, contributing to insulin resistance and the development of type 2 diabetes (McLaughlin et al., 2007).

Adipocyte differentiation and adipogenesis

White adipose tissue (WAT) is composed of various cell types, including adipocytes, endothelial cells, macrophages, and fibroblasts. Adipocytes, the cells specialized in fat storage, originate from fibroblast-like mesenchymal stem cells through a process known as adipogenesis. Initially, mesenchymal cells commit to the pre-adipocyte lineage, which subsequently differentiates into mature adipocytes. Adipogenesis is a complex process regulated by transcription factors, specifically CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor gamma (PPAR-γ) (Burgermeister et al., 2003; Gupta & Gupta, 2024).

During the first stage of adipogenesis, cyclic AMP (cAMP) induces C/EBP proteins. In the second stage, C/EBP-β and C/EBP-γ promote the expression of C/EBP-α and PPAR-γ, which are essential for differentiation. C/EBP-α and PPAR-γ mutually enhance each other's expression, driving the transcription of genes involved in lipid and glucose metabolism, thereby imparting insulin sensitivity to the adipocyte (Lafontan, 2012).

Adipocyte dysfunction and insulin resistance

Adipose tissue dysregulation is a significant contributor to insulin resistance. Although skeletal muscles account for 80% of glucose absorption and only 3% is taken up by adipose tissue, their glucose uptake is crucial for maintaining overall insulin sensitivity and glucose tolerance (Barazzoni et al., 2018). This process is essential for regulating energy balance and metabolic homeostasis. The storage of triglycerides in adipocytes involves several critical steps, starting with the activation of lipoprotein lipase (LPL). LPL hydrolyses triglycerides from circulating lipoproteins into free fatty acids, which are then taken up by adipocytes and re-esterified to form triglycerides for storage. This re-esterification process is closely linked to glucose metabolism, since glucose supplies the glycerol backbone necessary for triglyceride formation. Additionally, the translocation of the insulin-responsive glucose transporter GLUT4 to the adipocyte membrane is vital for glucose uptake into the cell, which further supports the synthesis and storage of triglycerides (Shepherd PR, 1999). Proper functioning of these mechanisms is vital for adipocyte health and metabolic homeostasis. The reduced uptake of glucose in adipose tissue due to impaired GLUT4 function disrupts normal lipid storage, leading to an overflow of fatty acids into the bloodstream. This results in ectopic fat deposition, inflammation, and systemic insulin resistance, which are key contributors to metabolic disorders such as type 2 diabetes and cardiovascular disease (Shepherd PR, 1999).

Moreover, the overexpression of GLUT4 specifically in adipocytes has been shown to lower fasting blood glucose levels and improve glucose tolerance (Sheperd PR,1999). This effect is linked to the upregulation of lipogenic genes, which are activated in response to increased glucose uptake, demonstrating the intricate relationship between glucose and lipid metabolism (Carvalho et al., 2005). The induction of lipogenic pathways in response to enhanced glucose availability highlights the role of adipocytes not just as passive storage units for triglycerides, but as active regulators of metabolic health. This interconnectedness between glucose and lipid metabolism in adipocytes emphasizes the impact that adipocyte function has on overall metabolic homeostasis and the potential consequences of its dysregulation (Herman et al., 2012).

Adipose tissue-signalling and functions

GLUT4 and glucose homeostasis

Glucose transporters (GLUTs) are integral membrane proteins that facilitate glucose transport across cellular membranes. Their expression and function are crucial for maintaining glucose homeostasis and vary across different tissues. Among these transporters, GLUT1 is widely distributed in tissues requiring constant glucose

supply, such as the blood-brain barrier and erythrocytes (Koch H, 2019). GLUT2, primarily expressed in the liver, pancreas, and kidneys, plays a key role in glucose sensing and regulation, facilitating glucose uptake and release based on metabolic needs (Thorens, 2015). GLUT3, found predominantly in neurons and the placenta, ensures adequate glucose transport in these metabolically active tissues (Simpson et al., 2008). GLUT4, which is central to this thesis, is the primary glucose transporter in adipose tissue and skeletal muscle, where it plays a critical role in insulin-regulated glucose uptake (Bryant et al., 2002).

Following a meal, elevated blood glucose levels stimulate the pancreas to secrete insulin into the bloodstream. Insulin then binds to its receptor on the surface of adipocytes, triggering a cascade of intracellular signalling events (Jian Sun et al., 1993). This binding activates insulin receptor substrates (IRS), which in turn activates phosphoinositide 3-kinase (PI3K). PI3K catalyses the conversion of phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). The accumulation of PIP3 facilitates the recruitment and activation of protein kinase B (Akt) (Boucher et al., 2014; Jian Sun et al., 1993) (Figure 1).

Akt phosphorylates several downstream targets, including Akt substrate of 160 kDa (AS160) (Kane et al., 2002). The phosphorylation of AS160 is crucial for the translocation of GLUT4 storage vesicles (GSVs) to the plasma membrane, thereby increasing GLUT4 availability on the cell surface and enhancing glucose uptake into the adipocyte (Jian Sun et al., 1993; Stenkula et al., 2010). This process is tightly regulated by the Ras-related in brain (Rab) proteins, which are GTPases associated with vesicle membranes (Sano et al., 2003). AS160 facilitates the transition of Rab proteins from their active GTP-bound state to the inactive GDP-bound state, which results in the retention of GLUT4 within intracellular storage compartments (Thurmond et al., 2003). When insulin stimulates the cell, Akt phosphorylates AS160, decreasing its GTPase-activating protein (GAP) activity. This enables Rab proteins to remain in their active GTP-bound state. This activation releases GSVs from intracellular storage sites and guides their movement toward the plasma membrane, where they undergo docking and fusion (Larance et al., 2005; Mîinea et al., 2005; Sano et al., 2003) (Figure1).

Once the GSVs reach the plasma membrane, the final step involves the docking and fusion of these vesicles with the membrane, a process that is critical for the proper functioning of glucose uptake (Bryant et al., 2002). The fusion event leads to the incorporation of GLUT4 into the membrane, thereby facilitating glucose entry into the cell (Leto & Saltiel, 2012) (Figure 1). Under basal conditions, GLUT4-containing vesicles exhibit extensive long-range movement along microtubule tracks, remaining ready to receive a fusion signal initiated by insulin. Upon insulin stimulation, there is a marked decrease in long-range GSV movement as more vesicles undergo fusion with the membrane (Rosen et al., 2021). This decrease reflects the efficient docking and fusion process, which ultimately results in a higher

number of GLUT4 transporters being available on the cell surface, thus enhancing glucose uptake (Bryant et al., 2002; Rosen et al., 2021).

GLUT4 undergoes continuous recycling within adipocytes. During fasting, GLUT4 is retained within GSVs, limiting glucose uptake into the cells(Leto & Saltiel, 2012)). Upon insulin stimulation, this recycling process is significantly amplified, leading to the translocation of approximately 50% of GLUT4 to the plasma membrane to enhance glucose uptake (Colosia et al., 2013; Hruby & Hu, 2015). This tightly regulated GLUT4 trafficking is essential for maintaining whole-body glucose homeostasis (Abel et al., 2001; Stenkula et al., 2010). Impaired glucose uptake can lead to hyperglycaemia and insulin resistance, as often seen in the adipose tissue of individuals with obesity and type 2 diabetes. These conditions are associated with reduced insulin signalling, impaired GLUT4 trafficking, and decreased GLUT4 protein levels, making GLUT4 translocation a rate-limiting step in glucose uptake (Abel ED, 2001; Stenkula et al., 2010).

The significance of AS160 in glucose metabolism is underscored by studies in which AS160 is mutated or deficient. In such models, GLUT4 translocation occurs even in the absence of insulin, leading to elevated basal glucose uptake. Furthermore, defects in AS160 phosphorylation are associated with impaired GLUT4 translocation and reduced glucose uptake, conditions that contribute to the development of insulin resistance and type 2 diabetes (Larance et al., 2005; Wang et al., 2013). AS160's role is not limited to insulin signalling alone. It is also regulated by other metabolic pathways, such as those involving AMP-activated protein kinase (AMPK). AMPK can phosphorylate AS160 independently of insulin, particularly in response to energy stress, thereby influencing GLUT4 translocation and glucose uptake in a manner that adapts to the cell's energy needs (Larance et al., 2005; Wang et al., 2013). This dual regulation highlights the versatility of AS160 as a molecular integrator of multiple signalling pathways that converge on GLUT4 trafficking (Mîinea et al., 2005).

The successful translocation of GLUT4 to the plasma membrane involves the dynamic reorganization of the actin cytoskeleton. Rab activation facilitates the translocation of GSVs, which is linked to the dynamic reorganization of the actin cytoskeleton. Under insulin stimulation, the actin cytoskeleton rearranges to support GLUT4 vesicle movement toward the plasma membrane. The efficient translocation of GLUT4 involves regulation by small GTP-binding proteins and myosin motors (Kanzaki et al., 2002). The reorganization of actin into mesh-like structures is critical for the successful translocation of GLUT4 and subsequent glucose uptake (Lim et al., 2015; Stall et al., 2014).

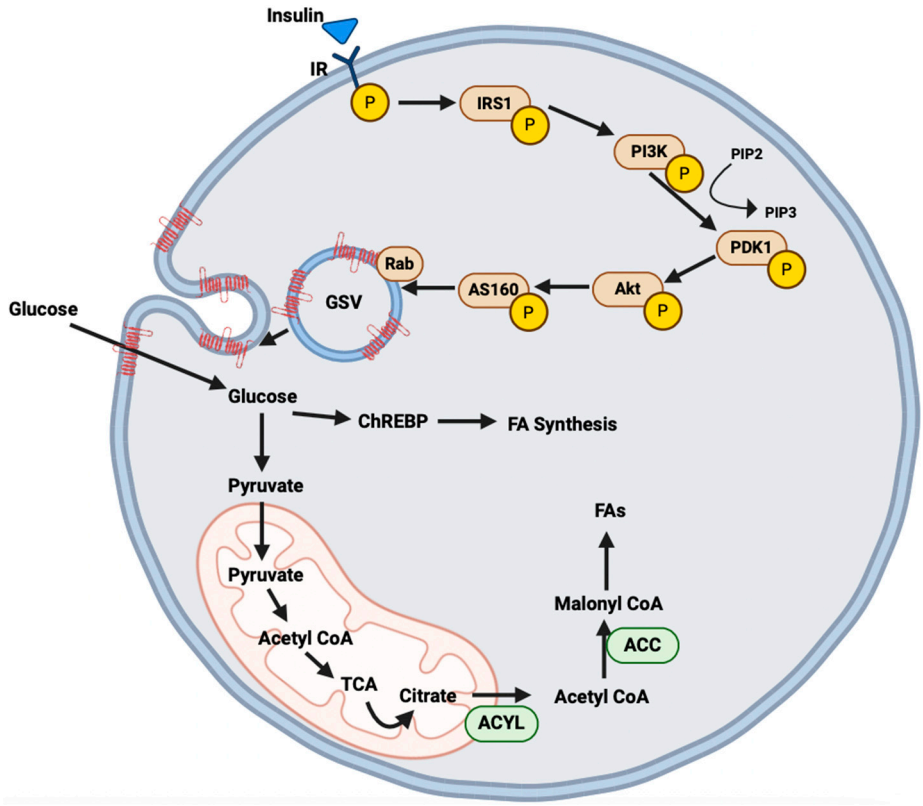

Figure 1. FA synthesis following Insulin stimulation.
Insulin binding to its receptor (IR) leads to phosphorylation (P) of IRS1, which activates PI3K. PI3K catalyzes the conversion of PIP2 to PIP3, which in turn activates PDK1 and Akt. Activated Akt phosphorylates AS160, promoting the fusion of GSVs with the plasma membrane and the translocation of GLUT4, facilitating glucose glucose uptake into the adipocyte. Intracellular glucose undergoes glycolysis, forming pyruvate, which is transported into the mitochondria and converted to acetyl-CoA. Acetyl-CoA enters the TCA cycle, generating citrate. Citrate is exported to the cytoplasm, where ACLY converts it back to acetyl-CoA. Acetyl-CoA is carboxylated by ACC to form malonyl-CoA, which FAS utilizes to elongate the carbon chain, ultimately producing palmitate, a 16-carbon saturated fatty acid. Insulin signalling activates ChREBP, enhancing the transcription of lipogenic genes and promoting fatty acid synthesis.

ECM provides structure and cell adhesion and prevents the cells from mechanical stress. In the adipose tissue, ECM and actin cytoskeleton remodel dynamically to accommodate for the hypertrophic and hyperplastic growth and angiogenesis (Catalán et al., 2012). In obesity, the remodelling is often linked to increased inflammation and the upregulation of pro-fibrotic signalling molecules like TGFβ (Williams et al., 2015; Yadav et al., 2011). This leads to impaired insulin signalling and glucose uptake, contributing to systemic insulin resistance (Bryant et al., 2002; Williams et al., 2015).

Fatty Acid synthesis

Mature adipocytes are characterized by a single large lipid droplet that occupies most of the cell, reducing the cytoplasm to a thin rim around the droplet. This lipid droplet serves as the storage site for fatty acids in the form of triglycerides (Lee et al., 2013).

Insulin plays a dual role in adipocytes: it suppresses lipolysis and promotes the uptake of glucose, which is vital not only for maintaining energy balance but also for facilitating triglyceride synthesis. This process is crucial for the storage of excess energy as fat, especially after meals when the body is in an energy-storing state (Lizunov et al., 2005). The increased intracellular glucose serves as a precursor for fatty acid (FA) synthesis. This process begins with the conversion of glucose to citrate through glycolysis, which is then converted to Acetyl-CoA via ATP-citrate lyase (ACLY) (Song et al., 2018). To initiate FA synthesis, Acetyl-CoA is converted to Malonyl-CoA, catalysed by acetyl-CoA carboxylase (ACC). Malonyl-CoA is subsequently used by fatty acid synthase (FAS) to elongate the carbon chain, producing palmitate, a 16-carbon saturated fatty acid (Brownsey et al., 2006) (Figure1).

Insulin plays a pivotal role in this lipogenic pathway by activating ACC and promoting the transcription of lipogenic genes, such as FAS, through carbohydrate response element-binding protein (ChREBP) activation, ensuring a steady supply of substrates for FA synthesis (Iizuka et al., 2004) (Figure 2). The synthesized fatty acids are then esterified with glycerol to form triglycerides, which are stored in lipid droplets within adipocytes (Iizuka et al., 2004). Proper regulation of FA synthesis is crucial for metabolic health; dysregulation can lead to ectopic fat deposition, insulin resistance, and metabolic disorders like type 2 diabetes and cardiovascular disease (Frayn KN., 2002).

Lipolysis

Lipolysis is a crucial metabolic process in adipocytes that involves the breakdown of triglycerides stored in lipid droplets into glycerol and free fatty acids. This process is essential for providing energy during periods of increased demand, such as exercise or caloric restriction (Bolsoni-Lopes & Alonso-Vale, 2015).

Insulin plays a key role in regulating adipocyte metabolism. In a low-insulin state, which typically occurs during fasting or other catabolic conditions, the body experiences an increased energy demand. Under these conditions, the release of catecholamines like adrenaline and noradrenaline stimulates β-adrenergic receptors on adipocytes. This activation triggers a signalling cascade that results in the production of cyclic AMP (cAMP) and the activation of protein kinase A (PKA). PKA then phosphorylates proteins like perilipin and HSL, facilitating the breakdown of triglycerides into free fatty acids and glycerol (Degerman et al., 2011) (Figure 1).

During fasting conditions, lipolysis is particularly important as it ensures a steady supply of free FA, which are released into the circulation to be used as an energy source by other tissues. Conversely, after food intake, insulin levels rise, and insulin acts to suppress lipolysis. This suppression occurs through the inhibition of HSL activity, mediated by insulin. Specifically, insulin signalling activates Akt, which subsequently activates phosphodiesterase 3B (PDE3B). PDE3B degrades cAMP, thereby reducing the activation of PKA and ultimately decreasing HSL activity. By inhibiting HSL, insulin prevents the excessive release of free fatty acids into the bloodstream and instead promotes their storage as triglycerides within the adipocytes (Yang & Mottillo, 2020).

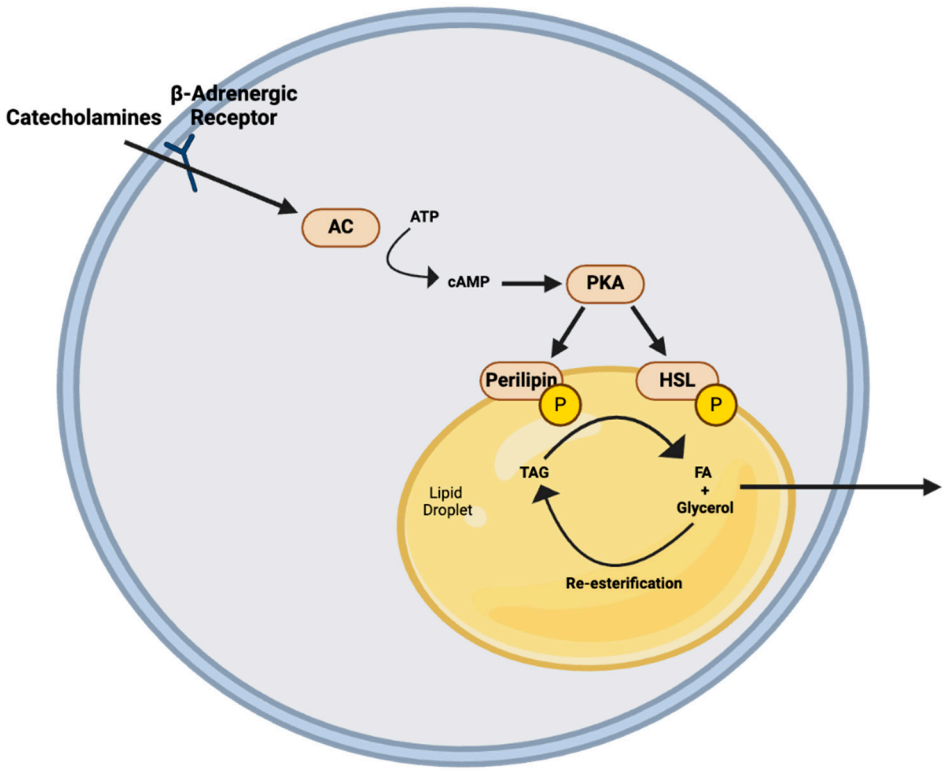

Figure 2. Lipolysis regulation.
Under energy demand, binding of catecholamines to the β-adrenergic receptor activates adenylate cyclase (AC), increasing cAMP levels. This activates PKA, which phosphorylates perilipin and HSL. Phosphorylated perilipin and HSL facilitate the breakdown of triglycerides (TAG) in lipid droplets into free fatty acids (FA) and glycerol. The FAs can be re-esterified or released from the cell for energy production.

Adipocyte models and experimental approaches in GLUT4 trafficking research

Adipocyte cell models

Mouse cells models are widely used to study GLUT4 trafficking due to their genetic similarity to humans and the easy genetic manipulation. In mice, GLUT4 trafficking follows a similar insulin-dependent mechanism as in humans (Bryant et al., 2002). However, mice have a higher basal metabolic rate relative to their body mass compared to humans, which can lead to a more rapid clearance of glucose from the bloodstream and contribute to a relatively higher insulin sensitivity (Ruan & Dong, 2016; Speakman, 2005).

Research in mice has provided valuable insights into the molecular mechanisms of GLUT4 trafficking. Knockout studies of key proteins in the insulin signalling pathway, such as IRS, PI3K, and Akt, have underscored their essential roles in GLUT4 translocation (Rosen ED, 2000). Mouse models of obesity and diabetes, induced by high-fat diets or genetic modifications, have demonstrated impaired GLUT4 trafficking, mirroring human conditions (Bryant et al., 2002). Interestingly, mice have compensatory mechanisms to preserve glucose homeostasis even when they develop insulin resistance (J. Kim et al., 2000). This compensation involves adaptations such as upregulation of GLUT1, which can facilitate glucose uptake in a manner less dependent on insulin (J. Kim et al., 2000). Furthermore, mice also increase their reliance on oxidative metabolism, which allows them to utilize glucose more efficiently through pathways that are less affected by insulin resistance (Kahle et al., 2013; Ruan & Dong, 2016).

Moreover, while human subcutaneous fat generally has neutral or protective effects on metabolism, excess visceral fat is linked to metabolic risks. In mice, visceral fat is primarily stored in peri-gonadal depots, which differ functionally from human visceral fat. Additionally, mouse visceral adipocytes are larger than subcutaneous ones, a pattern opposite to humans (Börgeson et al., 2022). These adaptive mechanisms in mice help to mitigate the effects of insulin resistance, allowing them to better maintain normal blood glucose levels compared to humans. In humans, similar levels of insulin resistance often result in more severe metabolic dysfunction, such as higher blood glucose levels and a greater risk of developing type 2 diabetes (Heydemann A., 2016; Kahle et al., 2013). These differences underscore the need to carefully consider species-specific characteristics when interpreting findings from mouse models and applying them to human health research (Heydemann A., 2016).

Most of the current knowledge of GLUT4 trafficking has relied on cells differentiated *in vitro*, such as 3T3L1 adipocytes, these models present limitations (Vogel A, 1973). While *in vitro* adipocytes have well established protocols for

differentiation, offering a uniform population of cells and are suitable for genetic manipulation they exhibit drawbacks. *In vitro* adipocytes contain several small droplets, a characteristic more typical of brown adipocytes, rather than the single large fat droplet seen in mature white adipocytes in vivo (Dufau et al., 2021). This difference in lipid droplet morphology can impact the spatial arrangement of cellular components like the nucleus and organelles, potentially influencing cellular function. Moreover, 3T3L1 adipocytes, over passages, exhibit a metabolic decline, including reduced glucose uptake, energy consumption, and fatty acid re-esterification, leading to a state of metabolic inactivity and increased insulin resistance (Vogel A, 1973). Furthermore, *in vitro* adipocytes demonstrate a less favourable GLUT4/GLUT1 ratio compared to differentiated primary adipocytes, compromising their suitability for studying insulin-regulated glucose uptake. Additionally, *in vitro* cells need a period of culturing post-transfection to enable protein expression, leading to poor cell survival and declining functionality over time (Dufau et al., 2021; Sadowski et al., 1992).

Conversely, primary adipocytes are obtained directly from adipose tissue, so the closely resemble mature adipocytes *in vivo*, featuring a single large lipid droplet. They predominantly express GLUT4, accounting for 90% of glucose transporters, with GLUT1 making up the remaining 10% (Dufau et al., 2021). This resemblance to *in vivo* adipocytes provides a more physiologically accurate response to insulin, reflecting a more reliable model for studying insulin-regulated glucose uptake and the body's blood sugar regulation mechanisms. However, despite their physiological relevance, primary adipocytes have limitations (Rudich et al., 2003). The isolation process itself is technically demanding and can easy yield variable results. Furthermore, due to their *in vivo* origin, isolated adipocytes have a limited lifespan, which poses challenges for experiments such transfection processes (Rudich et al., 2003).

Modulation of gene and protein expression

Electroporation and viral transduction *ex vivo* are common approaches to generate transient ectopic protein expression independent of the adipocyte model used. Electroporation is a commonly used method for introducing genetic material into primary adipocytes. By applying an electrical field, electroporation temporarily disrupts the cell membrane, allowing nucleic acids to enter the cell (Potter H., 1988). However, the success of electroporation depends heavily on the optimization of parameters such as voltage, pulse duration, and cell density to balance transfection efficiency and cell viability (Gehl J., 2003). In primary adipocytes, the process can be particularly challenging due to potential GLUT4 degradation post-isolation and the inherent membrane fragility. Additionally, primary adipocytes are sensitive to physical and environmental stress, and the

electroporation process itself can exacerbate this sensitivity by applying an electrical field to create transient pores in the cell membrane (Gehl J., 2003).

Adenovirus-mediated transfection offers an efficient alternative for genetic manipulation in primary adipocytes. The ability of adenoviruses to infect both dividing and non-dividing cells can be advantageous for targeting differentiated, non-proliferative cell types like mature adipocytes, which don´t divide (Rauschhuber et al., 2012). Additionally, adenoviral vectors allow the delivery of long DNA sequences and has been widely used to study glucose uptake and lipid metabolism regulation. The main advantage of adenovirus-mediated transfection is its high efficiency and ability to transduce a broad range of cell types. However, the initial uptake of the viral vector depends on receptor-mediated endocytosis, which is not always efficient (Kreppel & Kochanek, 2008). Controlling the level and duration of gene expression can be challenging and requires careful experimental design. Additionally, primary adipocytes can be sensitive to viral transfection, affecting cell viability and functionality (Rauschhuber et al., 2012).

Adenoviral transduction has proven to be a versatile and effective tool in studying various aspects of adipocyte biology and metabolism, particularly in the context of glucose homeostasis and insulin sensitivity. For instance, one study demonstrated that ectopic expression of uncoupling protein 1 (UCP1) in epididymal fat using adenoviral vectors could reverse insulin and leptin resistance, improving glucose tolerance and decreasing food intake in both diet-induced and genetically obese mouse models. This highlighted the potential of targeting intra-abdominal fat tissue to modulate metabolic syndrome components via afferent-nerve signals (Yamada et al., 2006). Another study utilized adenoviral transduction to explore the role of p21, a cyclin-dependent kinase inhibitor, in adipocyte differentiation and hypertrophy. The absence of p21 led to impaired adipocyte differentiation and reduced adipose tissue mass, ultimately resulting in improved insulin sensitivity in high-fat diet-fed mice. This finding highlighted the dual role of p21 in promoting adipocyte differentiation and protecting against apoptosis, contributing to adipose tissue expansion and subsequent insulin resistance (Inoue et al., 2008). Lastly, adenoviral-mediated gene transfer of pre-proinsulin into adipocytes was shown to ameliorate hyperglycaemia in diabetic KKA(y) mice, a model of type 2 diabetes. The localized expression of insulin in adipose tissue significantly reduced blood glucose levels and improved glucose tolerance without altering serum insulin levels, suggesting a novel therapeutic approach for managing type 2 diabetes (Nagamatsu, Nakamichi, Ozawa, et al., 2001). Collectively, these studies demonstrate the utility of adenoviral transduction in manipulating gene expression within adipose tissue to study and potentially treat metabolic disorders.

Advancements in GLUT4 trafficking research

While the current adipocyte models have been valuable in advancing our understanding of GLUT4 trafficking and adipocyte biology, the limitations associated with each model underscore the need for better strategies that can better recapitulate the physiological characteristics and functionalities of mature adipocytes *in vivo.*

A significant advancement in this area is the use of HA-GLUT4-GFP constructs. This construct combines the human GLUT4 transporter with a hemagglutinin (HA) tag and a green fluorescent protein (GFP) reporter. The early work of Al-Hasani and colleagues demonstrated that adenovirus-mediated transfection by electroporation could be used to express HA-GLUT4 in rat adipocytes. This method not only enhanced the visibility of GLUT4 trafficking but also offered a means to manipulate and study the role of other proteins in this process. For instance, by coexpressing dynamin mutants with HA-GLUT4, they showed that dynamin is crucial for GLUT4 endocytosis, with mutations leading to altered surface levels of GLUT4 in both basal and insulin-stimulated states (Al-Hasani et al., 1998).

In 2011, Dawson et al. used this system to track GLUT4 localization and trafficking in real-time within primary rat adipose cells. The HA tag facilitated GLUT4 immunostaining, while the GFP provided a fluorescent signal detectable under fluorescence microscopy. Their study showed that the C-terminal GFP-tagged GLUT4 mirrors the trafficking behaviour of endogenous GLUT4, supporting its use in high-throughput assays to screen potential anti-diabetic drugs (Dawson et al., 2001).

Further research has applied adenoviral vectors to compare the trafficking patterns of different glucose transporters, such as GLUT4 and GLUT1, in adipose cells. A common method used in genetic studies is the AdEasy system. This system facilitated the creation of recombinant adenoviruses, making it highly efficient for generating essential high-titer viral vectors (Luo et al., 2007). The AdEasy system involves a two-step process. First, a shuttle vector containing the gene of interest, such as GLUT4 or GLUT1, undergoes homologous recombination with an adenoviral backbone in bacteria. This step integrates the gene into the viral genome. After successful recombination, the recombinant adenoviral DNA is linearized and transfected into a packaging cell line, such as HEK293 cells, which produces the viral particles (Luo et al., 2007).

Despite the successes in using adenoviral transduction to study GLUT4 in adipocytes, challenges remain due to variability in GLUT4 trafficking behaviours across different cell types, such as 3T3-L1 adipocytes and CHO cells (J. Murettaet al., 2007). While constructs like HA-GLUT4-GFP have significantly advanced our understanding, these discrepancies highlight the necessity of optimized models to ensure accurate results (Dawson et al., 2001; Nagamatsu, Nakamichi, Ohara-Imaizumi, et al., 2001). Additionally, variations in the efficiency and impact of adenovirus-mediated transduction can influence gene expression control and cell

viability, further emphasizing the need for robust experimental approaches (J. Muretta et al., 2007).

Total internal reflection fluorescence microscopy

In primary white adipocytes, the central lipid droplet occupies 99% of the cell's volume, pushing all intracellular compartments to the cell periphery, adjacent to the plasma membrane. Most GLUT4 in white adipocytes is stored in GLUT4 storage vesicles (GSVs), located close to the plasma membrane (Martin et al., 2006). Total Internal Reflection Fluorescence (TIRF) microscopy is well-suited for studying adipocytes because it selectively illuminates a small volume near the cell membrane, minimizing light scattering caused by the large central lipid droplet (Lizunov et al., 2005; Wasserstrom et al., 2018). TIRF achieves this by using an incident light beam that penetrates just a few nanometres into the sample, creating an evanescent wave that excites only the fluorophores near the plasma membrane (Fish, 2022; Poulter NS, 2015).

This technique enhances the signal-to-noise ratio, making it particularly effective for visualizing dynamic processes such as membrane protein trafficking. By limiting excitation to the vicinity of the cell membrane, TIRF microscopy reduces background fluorescence, phototoxicity, and photobleaching, thereby preserving cell viability during extended imaging sessions. These features make TIRF microscopy an excellent tool for live-cell imaging, allowing researchers to capture rapid and dynamic events at the cell membrane with minimal photodamage (Poulter NS, 2015). In adipocyte research, this allows the observation of insulin-stimulated GLUT4 translocation, vesicle fusion, and other membrane-associated events in living cells. Visualizing these processes in real-time is crucial for our understanding of the molecular mechanisms underlying adipocyte function and metabolic regulation (Rao et al., 2022).

The characteristics of TIRF microscopy make it ideal for studying the rapid and dynamic events of GLUT4 trafficking at the primary adipocyte plasma membrane in response to insulin stimulation (Lizunov et al., 2005; Rao et al., 2022). Consequently, we used TIRF to evaluate GLUT4 trafficking in isolated primary adipocytes.

Materials and methods

In this section, I will summarize the methods used in my research. A more detailed description can be found in the manuscripts.

Adenoviral transduction

Adenovirus transduction is a highly efficient method for manipulating protein levels in cells by introducing specific adenovirus expression vectors. It offers several advantages, including the ability to infect both dividing and non-dividing cells, which is particularly useful for studying primary adipocytes.

In my research, we used a human GLUT4 adenoviral vector with modifications to include a hemagglutinin (HA) tag in the first exofacial loop, and a green fluorescent protein (GFP) tag at the C-terminal end, based on established protocols (Dawson et al., 2001; Quon et al., 1994).

The method involves direct injection of recombinant adenovirus into the epididymal fat pads of male mice, or parametrial fat pads in females, to ensure targeted expression of GLUT4. Mice are first anesthetized. A small incision, approximately 1 cm in length, is made along the midline of the lower abdomen to reach the fat pads. The fat pads are then gently exteriorized and placed on the peritoneum to administer the adenovirus, at concentrations ranging from $1x10^8$ to $1x10^{11}$ pfu/ml, directly into one or both fat pads. Injections are delivered in 50-100 µl of phosphate-buffered saline (PBS) across multiple sites within the fat pad (5-10 µl per site) to ensure even distribution of the virus. As experimental controls, the contralateral fat pad can receive an equivalent volume of PBS without the virus. After injections, the fat pads are repositioned into the abdominal cavity, and the incision is closed. This approach allows for the localized expression of the GLUT4 protein, which can be subsequently analysed. Mice are euthanized at various time intervals post-transduction, and the fat pads are harvested for tissue homogenization, immunoblotting, RNA extraction, or isolation of adipocytes.

As mentioned in the introduction, the use of adenoviral transduction in adipose tissue has been well-documented. This method has been employed in various studies to manipulate and observe specific proteins within adipocytes, such as the role of UCP1 in glucose tolerance (Yamada et al., 2006), adenoviral-mediated expression of preproinsulin in diabetic mice (Nagamatsu, Nakamichi, Ozawa, et al., 2001) and

the role of p21 in adipocyte hypertrophy and obesity-induced insulin resistance (Inoue et al., 2008).

Mouse models

In this thesis, I conducted my research using primary adipocytes isolated from the epididymal fat pads of mice. While mouse adipose tissue may not be identical to human fat, the epididymal fat pad is one of the most studied fat depots in rodent models. It offers a reliable and relevant source of adipocytes for investigating the mechanisms of insulin action and GLUT4 trafficking. The use of this fat depot is advantageous due to its accessibility and the relative abundance of adipocytes, making it a practical and effective choice for experimental studies. Utilizing *in vivo* models, such as primary adipocytes isolated directly from mice, provides a more accurate representation of the unaltered adipocytes compared to traditional cell culture.

Isolation of Primary Adipocytes

To obtain primary adipocytes for this study, the epididymal fat pads were carefully excised from the mice. Following extraction, the adipose tissue was subjected to collagenase digestion, a method that enzymatically breaks down the extracellular matrix, allowing the release of individual adipocytes. By isolating these adipocytes immediately after their extraction from the mouse this approach helps to retain their physiological properties, which is critical for studying insulin-stimulated glucose uptake and the behaviour of GLUT4 *in vivo*.

Western Blotting

Western blotting is widely used for detecting and quantifying specific proteins in a sample. In my research, Western blotting was employed to assess the expression levels of GLUT4 and other key proteins involved in insulin signaling pathways in adipocytes.

The process started with the preparation of extracts from isolated adipocytes. Proteins were separated based on their molecular weight through SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). After separation, the proteins were transferred onto a nitrocellulose membrane, which served as a solid support for antibody binding. Once the proteins are immobilized on the membrane, the blot was incubated with specific primary antibodies that target the protein of interest, such as GLUT4, IRS1, or phosphorylated AS160. Following this, secondary antibodies conjugated with an enzyme like horseradish peroxidase were applied. These secondary antibodies bind to the primary antibodies and facilitated the detection of the target protein through a chemiluminescent reaction. The

resulting signal was visualized using a chemiluminescence imaging system, allowing for the quantification of protein expression levels. Western blotting was crucial for verifying the efficiency of adenovirus-mediated transduction and for confirming the presence of the tagged proteins.

RNA isolation and RT-qPCR

To examine gene expression of inflammatory markers we used reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The mRNA expression of inflammation markers TNFα and IL6 was measured by qPCR on reverse-transcribed RNA, following the procedure described by Kim et al. (J. I. Kim et al., 2019).

Glucose uptake assay

In this thesis, glucose uptake was measured to investigate the effectiveness of GLUT4 translocation in response to insulin stimulation in primary mouse adipocytes. By measuring how much glucose was absorbed by the adipocytes in response to insulin, we could evaluate the sensitivity to insulin.

Glucose uptake was measured using the method described by (Gliemann et al., 1984). In this procedure, adipocytes in suspension were incubated with or without insulin to observe the effects of insulin stimulation. After this incubation, radiolabeled D-14C(U)-glucose was added to the cell suspension to track glucose uptake. To halt the uptake process, the cell suspension was centrifuged in microtubes containing dinonylphthalate oil, which facilitated the separation of the adipocyte pellet. The resulting cell pellet was then collected and dissolved in scintillation fluid. Glucose uptake was quantified by measuring the radioactive decay of the labeled glucose using scintillation counting, providing a precise measurement of how much glucose was absorbed by the adipocytes.

Immunofluorescence

In my research, immunostainng was used to detect HA-tagged GLUT4 on the surface of isolated adipocytes. After isolating isolation, adipocytes were incubated with primary antibodies that recognize the HA tag on GLUT4, allowing for specific labeling. Afterward, secondary antibodies conjugated with fluorescent dyes were used to bind to the primary antibodies.This setup enabled the detection of HA-GLUT4 using fluorescence microscopy.

The advantage of immunostaining lies in its ability to provide detailed spatial information about protein localization, distribution and expression levels, offering insights into how insulin affects GLUT4 trafficking to the cell surface. By

combining immunostaining with advanced imaging techniques like confocal and TIRF microscopy, high-resolution images can be obtained.

Confocal and TIRF microscopy

Confocal and TIRF microscopy are powerful imaging techniques used to study the localization and dynamics of proteins following immunofluorescence staining.

For confocal microscopy, a suspension of isolated adipocytes was placed onto slides. Brightfield imaging at 10X magnification was used initially to count total cell numbers, followed by fluorescence imaging to detect GFP-positive cells, indicating successful transduction with the HA-GLUT4-GFP construct.

For TIRF microscopy, adipocytes were also prepared in a cell suspension and transferred to a coverslip. This setup was placed in a glass chamber on a heated stage maintained at 37°C. The laser penetration depth was set to 180 nm to selectively illuminate the region close to the plasma membrane, capturing the trafficking of HA-GLUT4-GFP vesicles. Time-lapse imaging was conducted over a period of 2 minutes. To assess insulin response, 10 nM insulin was introduced during imaging, and recording continued for another 2 minutes.

Image analysis

Image analysis was critical for quantifying GSV dynamics and understanding their behaviour under different experimental conditions. The movement of GSVs was tracked using metrics such as Mean Square Displacement (MSD) to quantify the extent and pattern of vesicle movement.

Images were processed using ImageJ. Automated detection and tracking of GLUT4 vesicles were carried out using the Mosaic Particle Tracker 2D/3D plugin. The coordinates obtained from tracking were further analyzed using R statistical software to calculate parameters such as net displacement, total displacement, and the trajectory of GLUT4 vesicles. Data analysis involved statistical testing using GraphPad Prism 6 software. Images were processed using ImageJ software (version 1.46r), with the StackReg plugin employed to correct for any drift in the images during time-lapse recording. Automated detection and tracking of GLUT4 vesicles were carried out using the Mosaic Particle Tracker 2D/3D plugin, which enabled detailed analysis of vesicle movement within defined regions (35x35 or 10x10 μm squares).

The coordinates obtained from tracking were further analyzed using R statistical software to calculate parameters such as net displacement, total displacement, and the trajectory of GLUT4 vesicles. Data analysis involved statistical testing using GraphPad Prism 6 software, employing Student's t-test or ANOVA as appropriate.

Overall aim and specific aims

General Aim

The aim of this thesis is to understand the adipocyte function in normal insulin-sensitive state, which will be helpful to prevent the onset of insulin resistance, and later on T2D.

Specific aims

- To optimize an *in vivo* adenoviral transduction method for perigonadal white adipose tissue to investigate the mechanisms of GLUT4 trafficking in freshly isolated adipocytes under basal and insulin-stimulated conditions.
- To evaluate the impact of adenoviral transduction on adipose tissue insulin sensitivity, by comparing insulin-stimulated signalling and glucose uptake in transduced versus non-transduced adipocytes.
- To monitor GLUT4 vesicle movement in adipocytes from wild type and AS160 knockout mice, examining the differences in vesicle dynamics under basal and insulin-stimulated conditions.

Results and discussion

In vivo adenoviral transduction of perigonadal adipose tissue to assess GLUT4 trafficking in freshly isolated adipocytes.

Understanding the GLUT4 trafficking and insulin stimulation processes is essential for developing targeted therapies aimed at improving GLUT4 function and insulin sensitivity in adipocytes, thereby mitigating the metabolic disturbances associated with diabetes (Sadowski et al., 1992; Vogel A, 1973). While primary adipocytes better reflect a physiological context, these cells rapidly lose their endogenous GLUT4 protein content and differentiation markers during the required overnight culture, which is needed to allow ectopic GLUT4 protein expression. Factors like temperature, osmotic balance, and mechanical stress during processing can accelerate degradation, which increases with time post-isolation (Sadowski et al., 1992).

These limitations highlight the need for more physiologically relevant in vivo models to study GLUT4 trafficking. In response to the need of an improved *in vivo* model, my research adopts an approach using adenoviral infection *in vivo* to study GLUT4 trafficking in freshly isolated adipocytes.

In this paper, we aimed to optimize an *in vivo* adenoviral transduction method for perigonadal white adipose tissue to investigate GLUT4 trafficking in freshly isolated adipocytes under both basal and insulin-stimulated conditions.

Key findings:

- The method for *in vivo* adenoviral mediated gene expression works for GLUT4 studies.
- The method maintained the functional properties of adipocytes, as insulin response and GLUT4 dynamics were unaffected by the transduction process.
- AS160-deficient adipocytes exhibited impaired GLUT4 translocation, as insulin did not induce further GSV exocytosis.

Optimization of *in vivo* adenoviral transduction of perigonadal adipose tissue with HA-GLUT4-GFP

Our initial experiments focused on optimizing the adenoviral infection in epididymal white adipose tissue using a GFP-tagged control virus and a HA-GLUT4-GFP virus. We collected samples at 2, 4, and 6 days post-infection for immunoblotting to detect GLUT4 (Figure 3A). The highest expression levels were observed at day 2, with no detectable expression by day 6.

We then investigated the effect of different viral doses on HA-GLUT4-GFP expression. Different virus titers, as detailed in the figure legend, were used, and HA-GLUT4-GFP expression was analysed two days post-transduction. Immunoblots for GLUT4 and GFP (Figure 3B) showed a decrease in HA-GLUT4-GFP expression with lower viral doses, with no expression detectable at the lowest titer.

Considering that adenoviral transduction can induce inflammation, we measured levels of inflammatory markers tumour necrosis factor α (TNFα) and interleukin-6 (IL-6), in the transduced adipocytes at various time points (Figure 3C). Both PBS and adenovirus injections caused an inflammatory response on day 1, but the response was significantly larger with the adenovirus, declining over the next two days. By 48 hours post-infection, inflammation levels were minimal. This time frame provided a balance between maximal adenovirus expression and minimal inflammatory response, as confirmed by the reduced levels of TNF-α and IL-6 (Figure 3C). We determined that the infection time course was higest at day two and no longer detectable at day 6.

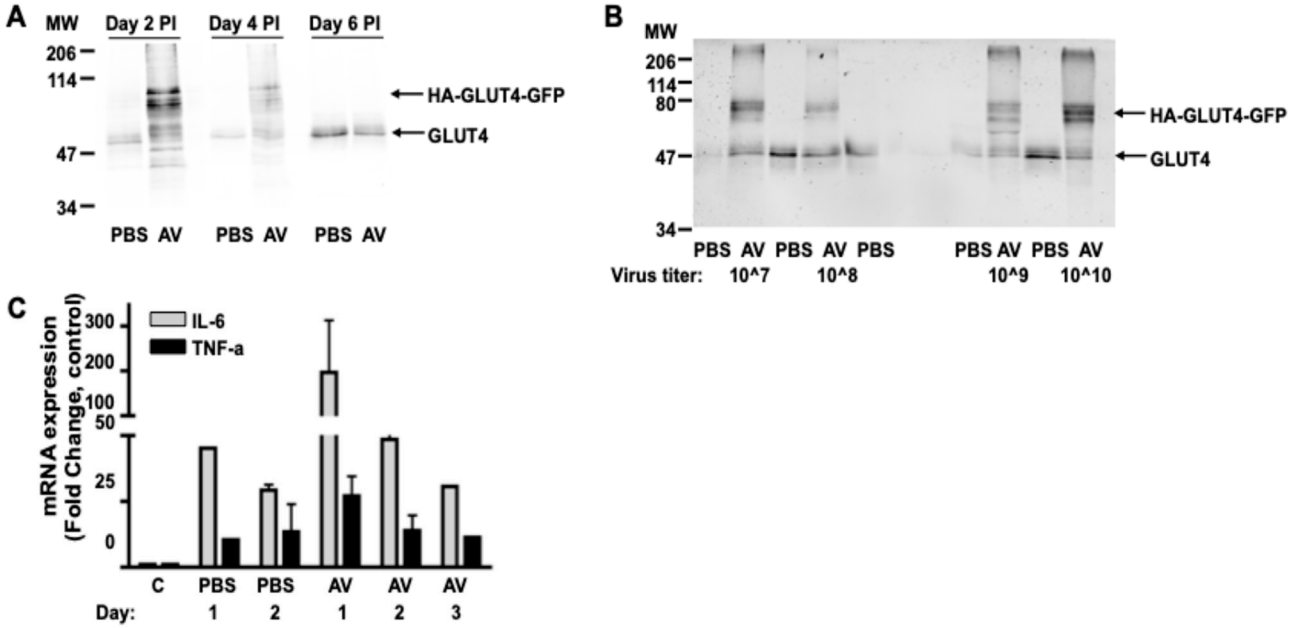

Figure 3. Perigonadal fat pads (male or female/epididymal or parametrial) of mice injected with adenovirus containing HA-GLUT4-GFP cDNA or PBS. **A)** Immunoblot for GLUT4 in samples collected at 2, 4, and 6 days post-infection (PI) with PBS (100 µl total volume) or adenovirus (AV) containing HA-GLUT4-GFP (titer 10^9 pfu). The highest expression of HA-GLUT4-GFP is observed on day 2, with a marked reduction by day 6. **B)** HA-GLUT4-GFP expression using varying viral titers (10^7 and 10^{10} pfu), analysed through GLUT4 immunoblot. Higher viral doses resulted in stronger expression, with minimal or no expression at the lowest titer. **C)** mRNA levels of inflammatory markers TNF-α and IL-6 at 1, 2, and 3 days post-infection, comparing PBS and AV-injected tissues. Fold changes were calculated by comparing 2^{-Ct} of injected fat pads with control, setting values for the control at 1. Both markers exhibited increased expression on day 1, with a more pronounced response in AV-injected samples, which decreased by day 3, indicating a balance between effective transduction and controlled inflammation, with minimal levels at 48 hours post-infection.

To assess the efficiency of transduction, we explored the optimal adenoviral dose for achieving the desired levels of HA-GLUT4-GFP expression for microscopy. Our goal was to ensure sufficient fluorescence intensity without causing signal saturation or interference (Figure 4A). Virus titers of 10^7 and 10^8 pfu were tested, using TIRF and confocal microscopy to visualize cells and measure transduction efficiency. Both titers produced similar numbers of GFP-positive adipocytes, with an average efficiency of 82 ± 4.4%. To verify the correct translocation of transduced HA-GLUT4-GFP to the cell surface upon insulin stimulation, we performed HA-staining on non-permeabilized adipocytes. After insulin treatment, there was an increase in the HA signal at the cell surface (Figure 4B), indicating successful insertion of GLUT4 into the plasma membrane.

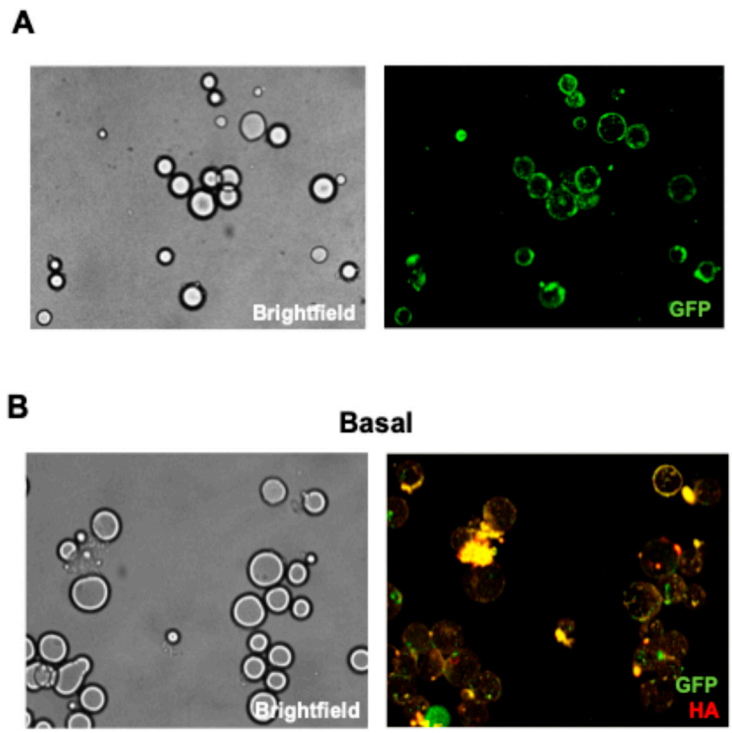

Figure 4. Localization of HA-GLUT4-GFP in isolated adipocytes under basal and insulin-stimulated conditions.

Adipocytes were isolated two days after transduction using titer as indicated and subjected to HA staining after stimulation with or without insulin (10 nM), 20 min. A) Representative image of isolated adipocytes examined under brightfield (10x) to determine total cell numbers and fluorescence light to determine GFP-positive cells. ~100 cells/field were evaluated, n=4 biological replicates. Efficiency of transduction was calculated as (fluorescent GFP-positive cells/total cells)x100. B) Representative image of basal a adipocytes marked for HA signal. Under basal conditions, the HA-GLUT4-GFP is predominantly intracellular, as indicated by the overlapping GFP and HA signals (Yellow).

Evaluation of Insulin Sensitivity and Glucose Uptake in Transduced Adipocytes

We conducted a series of experiments to ensure that virus-mediated transduction did not compromise overall adipose tissue responsiveness. To achieve this, we examined insulin signalling and glucose uptake in adipocytes isolated from transduced tissue. We also aimed to determine the necessary viral particle units (pfu) for efficient transduction. Varying amounts of a GFP-tagged control virus, specifically 1×10^8, 5×10^8, or 1×10^9 pfu, were injected into each fat pad, and the results were analysed using TIRF microscopy. The transduction efficiency, calculated as the ratio of GFP-positive cells to the total number of cells, showed a positive correlation with increasing pfu. We observed transduction efficiencies of approximately 11%, 25%, and 27% with increasing viral doses (1×10^8, 5×10^8, or 1×10^9 pfu per fat pad). Considering the minimal difference in efficiency between 5×10^8 and 1×10^9 pfu, we chose to use 5×10^8 pfu for subsequent analyses.

To assess adipocyte function and systemic metabolism in cells isolated from mice undergoing adenoviral transduction, we measured insulin responsiveness by examining intracellular signalling and glucose uptake. Western blot analysis revealed that insulin-stimulated phosphorylation of IRS (Tyr612), PKB (Ser473), and AS160 (Thr642) was similar in cells from non-transduced and transduced fat pads (Figure 5A). Quantitative measurements confirmed that the virus-transduced cells displayed a response to insulin stimulation (10 nM) comparable to PBS-treated controls, indicating that the insulin-dose response was preserved in cells isolated from either control (non-injected) or fat pads injected with either PBS or virus (Figure 5A).

Supporting the western blot analysis, glucose uptake was evaluated in three different cell groups: wild type (Non-treated), a negative control (PBS), and virus-transduced cells (GFP) (Figure 5B). These groups were tested under three conditions: basal (no stimulation), 0.1 nM insulin, and 1 nM insulin. The results demonstrated an insulin dose-dependent increase in glucose uptake across all groups. Notably, the virus-transduced cells exhibited a glucose uptake pattern similar to both the normal and negative control groups. This consistency in response validates the effectiveness of our viral transduction method for studying the glucose trafficking pathway without impairing insulin response or glucose transport.

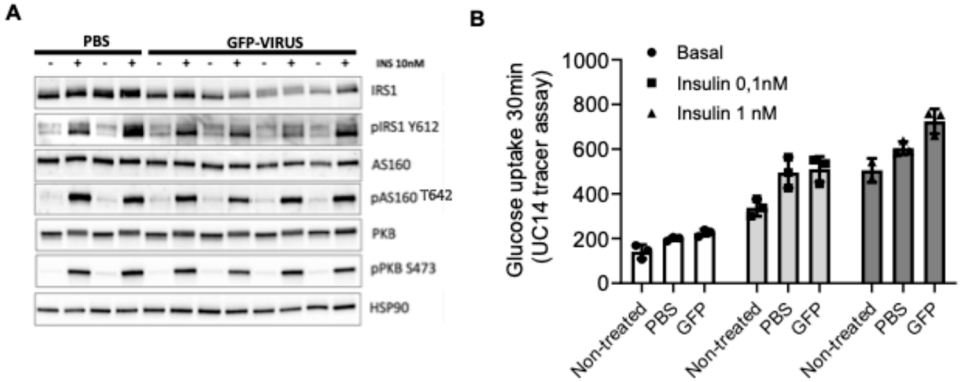

Figure 5. Epididymal fat pads from male mice transduced with increasing virus doses ($1x10^8$, $5x10^8$, $1x10^9$ pfu/fat pad).
A) Immunoblots of adipocytes 2 days post-transduction injected with PBS or GFP-tagged virus stimulated **with** or without insulin 10 nM for 30 min. The blots show levels of IRS1, phosphorylated IRS1 (pIRS1 Y612), AS160, phosphorylated AS160 (pAS160 T642), PKB, and phosphorylated PKB (pPKB S473). Quantification shows that insulin stimulation enhances the phosphorylation of these signalling proteins in both PBS and GFP-virus treated cells, indicating intact insulin signalling post-transduction. n=3 biologic replicates/condition. HSP90 used as a loading control. **B)** Glucose uptake of isolated adipocytes from non-treated, PBS-injected, and GFP-virus injected fat pads that were incubated under basal conditions, with 0.1 nM insulin, or with 1 nM insulin for 30 minutes and subjected to tracer glucose uptake assay. Glucose uptake increases in an insulin dose-dependent manner. Data are expressed as uptake/cell suspension volume, n=3 independent experiments.

Analysis of GLUT4 Vesicle Movement by TIRF in Wild Type and AS160-Deficient Adipocytes

AS160 inhibits the release of vesicles containing GLUT4 from intracellular storage compartments. Previous studies have shown that in the absence of AS160, there is an increase in the basal level of GLUT4 at the cell surface, indicating a critical role of AS160 in retaining GLUT4 within intracellular compartments under non-stimulated conditions. For instance, AS160-deficient adipocytes exhibit increased GLUT4 translocation to the plasma membrane even in the absence of insulin stimulation (Lansey et al., 2012). Building on this, we employed *in vivo* adenoviral transduction of adipose tissue with HA-GLUT4-GFP to examine GLUT4 trafficking using TIRF microscopy in adipocytes from wild type and AS160 knockout mice. By transducing a HA-GLUT4-GFP adenovirus into adipose tissue of AS160 knock-out mice, we aimed to observe GLUT4 dynamics under both basal and insulin-stimulated conditions in a setting where GSVs translocation is compromised (Data not shown).

HA-GLUT4-GFP expression levels were similar in wild-type and AS160-deficient transduced adipose tissue (Figure 6A). Quantitative analysis revealed that wild type

cells displayed the highest net displacement of GLUT4 vesicles under basal conditions, while insulin-stimulated wild-type and AS160-deficient cells had significantly lower and similar displacement levels (Figure 6B). The analysis of vesicle movement frequencies indicated that in unstimulated wild type cells, the tracks were the longest, with a peak mean square displacement (MSD) of 0.35 μm². In contrast, insulin-stimulated wild type cells exhibited shorter tracks, with MSD values ranging from 0.1 to 0.35 μm². AS160-deficient cells had a more noticeable shift towards shorter tracks, peaking at 0.1 μm². This suggests that AS160 is essential for the regulation of GLUT4 vesicle movement in response to insulin.

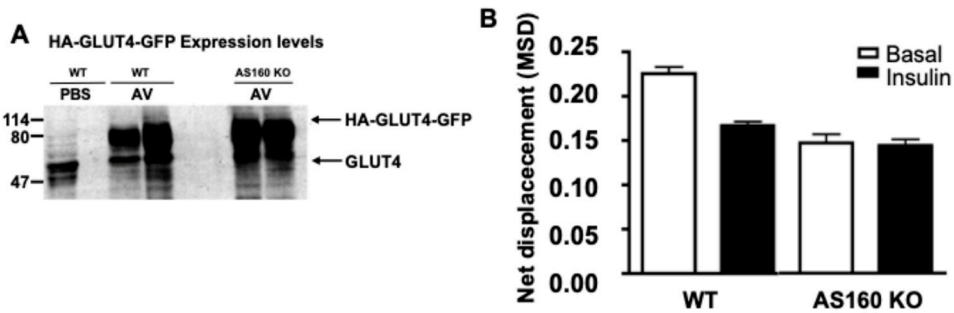

Figure 6. Perigonadal adipocytes from male wild type and AS160 knockout mice.
A) Immunoblot processed for HA-GLUT4-GFP and endogenous GLUT4 in wild type (WT) and AS160 knockout (KO) adipose tissue. The adipose tissues were transduced with adenovirus (AV: 10^7 pfu, AV2: 10^8 pfu)) carrying HA-GLUT4-GFP or treated with phosphate-buffered saline (PBS) as a control. The molecular weight markers are indicated on the left (114, 80, and 47 kDa). HA-GLUT4-GFP expression levels are similar in both WT and AS160 KO transduced tissues, while the PBS sample shows no HA-GLUT4-GFP expression. **B)** Mean square displacement (MSD) of GLUT4 vesicles in WT and AS160 KO adipocytes injected with adenovirus with HA-GLUT4-GFP under basal and insulin-stimulated conditions. Movies were taken using TIRF two days post-injection. Time-lapse movies were recorded at a rate of one frame every two seconds over a span of two minutes. This was performed at 60X magnification with a laser penetration depth of 180 nm, using excitation at 488 nm and emission at 510 nm, to capture live images of both WT and AS160 KO adipocytes under basal conditions and after the addition of 10 nM insulin. Representative data for one of two experiments are shown. WT basal cells exhibit the highest net displacement, which significantly reduces upon insulin stimulation. Both basal and insulin-stimulated AS160 KO cells show lower and similar MSD levels, similar to insulin-stimulated WT cells.

Concluding remarks

Understanding the mechanisms that regulate GLUT4 retention and release to the cell surface is essential for controlling glucose uptake. Traditional studies have often used cultured adipose cell models, such as 3T3-L1 adipocytes, which differ significantly from primary adipocytes in glucose transporter expression and distribution. These differences can impact the interpretation of GLUT4-specific trafficking events. Primary adipocytes, while more physiologically relevant, lose

their endogenous GLUT4 and differentiation markers quickly during overnight culture.

To overcome these limitations, we refined an in vivo adenoviral transduction method to express tagged GLUT4 in freshly isolated adipocytes, suitable for live-cell imaging. This approach has been previously applied to various aspects of adipocyte biology, including glucose tolerance, insulin sensitivity, differentiation, and hyperglycemia management. By optimizing the timing post-virus induction to 2 days and adjusting the virus dose to 5×10^8 pfu, we achieved effective transduction with minimal inflammation and optimal fluorescence for TIRF microscopy.

Our findings demonstrate that the adenoviral transduction technique preserves insulin response and GLUT4 dynamics in adipocytes. Both basal and insulin-stimulated glucose uptake were comparable in adipocytes from mice injected with PBS or adenovirus and those from non-surgery mice (67). This confirms the method's reliability for studying GLUT4 translocation, overcoming the common issue of impaired insulin response in primary adipocyte culture.

We applied this method to observe live-cell GLUT4 trafficking in WT and AS160 KO mice adipocytes. In agreement with previous studies, we found that WT adipocytes displayed significant GLUT4 movement under basal conditions, which decreased with insulin stimulation. In AS160-deficient adipocytes, GLUT4 trafficking was impaired under basal conditions, similar to insulin-stimulated WT cells. These results support earlier findings in AS160-deficient 3T3-L1 adipocytes, where AS160 deficiency led to increased GLUT4 translocation to the plasma membrane without insulin.

Despite its complexity, the in vivo adenoviral transduction model offers substantial advantages over traditional cell culture models, enabling real-time studies of GLUT4 dynamics in a physiologically relevant setting. Our *in vivo* adenoviral transduction technique has opened new avenues for studying protein trafficking in a physiologically relevant context. Future studies could focus on exploring the dynamics of GLUT4 in different settings such male versus female mice, diet interventions, transgenic mouse models, dual reporters or different adipose tissues, such as visceral versus subcutaneous fat, to elucidate their distinct roles in insulin sensitivity and metabolic disease progression.

Popular summary

La obesidad y la diabetes tipo 2 están aumentando a tasas alarmantes en todo el mundo, presentando desafíos significativos para la salud pública. Un componente clave en estas condiciones es GLUT4, una proteína que transporta glucosa a las células en respuesta a la insulina. El funcionamiento adecuado de GLUT4 es crucial para mantener niveles saludables de azúcar en la sangre y la salud metabólica en general. Sin embargo, estudiar el comportamiento de GLUT4 en modelos de laboratorio tradicionales tiene sus limitaciones, ya que estos modelos a menudo no imitan con precisión el entorno complejo de los tejidos vivos.

Para abordar esto, en esta tesis exploro los mecanismos moleculares que influyen en la acción de la insulina, con un enfoque en el tráfico de GLUT4 en adipocitos. Dadas las limitaciones de los modelos tradicionales *in vitro*, desarrollamos un método de transducción adenoviral *in vivo* para expresar HA-GLUT4-GFP en tejido adiposo blanco perigonadal. Este enfoque nos permitió observar la dinámica de GLUT4 en adipocitos recién aislados bajo condiciones basales y estimuladas por insulina utilizando microscopía TIRF.

Esta investigación también investiga el comportamiento de los adipocitos de ratones con y sin una proteína llamada AS160, que juega un papel crucial en la retención de GLUT4 dentro de las células cuando no hay insulina presente. Nuestras observaciones revelaron que en ratones normales, la insulina causaba el desplazamiento de GLUT4 a la superficie celular, facilitando la captación de glucosa. Sin embargo, en ratones que carecían de AS160, GLUT4 ya estaba en la superficie celular incluso sin estimulación de insulina, destacando la importancia de AS160 en la regulación de la glucosa.

Este método proporciona una imagen más precisa y detallada del comportamiento de GLUT4 en tejidos vivos, ofreciendo valiosos conocimientos sobre los mecanismos subyacentes a la acción de la insulina y la captación de glucosa. Estos hallazgos podrían llevar a mejores estrategias terapéuticas para manejar y tratar enfermedades metabólicas como la diabetes y la obesidad.

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References

Abel, E., Peroni O, Kim, J., Boss, O., Hadro, E., Minnemann, T., Shulman, G., & Khan, B. (2001). Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature*, *409*(6821), 729–733.

Al-Hasani, H., Hinck, C. S., & Cushman, S. W. (1998). Endocytosis of the glucose transporter GLUT4 is mediated by the GTPase dynamin. *Journal of Biological Chemistry*, *273*(28), 17504–17510. https://doi.org/10.1074/jbc.273.28.17504

Arner, P. (1998). Not all fat is alike. *The Lancet*, *351*(9112), 1301–1302. https://doi.org/10.1016/S0140-6736(05)79052-8

Barazzoni, R., Gortan Cappellari, G., Ragni, M., & Nisoli, E. (2018). *Insulin resistance in obesity-An overview of fundamental alterations*. doi:10.1007/s40519-018-0481-6

Berg, A. H., & Scherer, P. E. (2005). Adipose tissue, inflammation, and cardiovascular disease. In *Circulation Research* (Vol. 96, Issue 9, pp. 939–949). https://doi.org/10.1161/01.RES.0000163635.62927.34

Bolsoni-Lopes, A., & Alonso-Vale, M. I. C. (2015). Lipolysis and lipases in white adipose tissue – An update. In *Archives of Endocrinology and Metabolism* (Vol. 59, Issue 4, pp. 335–342). Sociedade Brasileira de Endocrinologia e Metabologia. https://doi.org/10.1590/2359-3997000000067

Börgeson, E., Boucher, J., & Hagberg, C. E. (2022). Of mice and men: Pinpointing species differences in adipose tissue biology. In *Frontiers in Cell and Developmental Biology* (Vol. 10). Frontiers Media S.A. https://doi.org/10.3389/fcell.2022.1003118

Boucher, J., Kleinridders, A., & Ronald Kahn, C. (2014). Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harbor Perspectives in Biology*, *6*(1). https://doi.org/10.1101/cshperspect.a009191

Brownsey, R., Boone, A., Elliott, J., Kulpa, J., & Lee, W. (2006). Regulation of acetyl-CoA carboxylase. *Biochem Soc Trans*, *34*(2), 223–227.

Bryant, N., Govers, R., & James, D. (2002). Regulated transport of the glucose transporter GLUT4. *Nat Rev Mol Cell Biol.*, *3*(4), 267–277. doi: 10.1038/nrm782. PMID: 11994746.

Burgermeister, E., Tencer, L., & Liscovitch, M. (2003). Peroxisome proliferator-activated receptor-gamma upregulates caveolin-1 and caveolin-2 expression in human carcinoma cells. *Oncogene*, *22(25)*, 3888–3900. doi:10.1038/sj.onc.1206625

Carvalho, E., Kotani, K., Peroni, O. D., & Kahn, B. B. (2005). Adipose-specific overexpression of GLUT4 reverses insulin resistance and diabetes in mice lacking GLUT4 selectively in muscle. *Am J Physiol Endocrinol Metab*, *289*, 551–561. https://doi.org/10.1152/ajpendo.00116.2005.-Adipose

Catalán, V., Gómez-Ambrosi, J., Rodríguez, A., & Frühbeck, G. (2012). Role of extracellular matrix remodelling in adipose tissue pathophysiology. Relevance in the development of obesity. In *Histology and Histopathology* (Vol. 27, Issue 12, pp. 1515–1528). https://doi.org/10.14670/HH-27.1515

Colosia, A. D., Palencia, R., & Khan, S. (2013). Prevalence of hypertension and obesity in patients with type 2 diabetes mellitus in observational studies: A systematic literature review. *Diabetes, Metabolic Syndrome and Obesity*, *6*, 327–338. https://doi.org/10.2147/DMSO.S51325

Dawson, K., Aviles-Hernandez, A., Cushman, S. W., Malide, D., Dawson, K., & Malide, D. (2001). Insulin-regulated trafficking of dual-labeled glucose transporter 4 in primary rat adipose cells. *Biochemical and Biophysical Research Communications*, *287*(2), 445–454. https://doi.org/10.1006/bbrc.2001.5620

Degerman, E., Ahmad, F., Chung, Y. W., Guirguis, E., Omar, B., Stenson, L., & Manganiello, V. (2011). From PDE3B to the regulation of energy homeostasis. In *Current Opinion in Pharmacology* (Vol. 11, Issue 6, pp. 676–682). Elsevier Ltd. https://doi.org/10.1016/j.coph.2011.09.015

Dufau, J. emy, Shen, J. X., Couchet, M., de Castro Barbosa, T., Mejhert, N., Massier, L., Griseti, E., Mouisel, E., Amri, E. Z., Lauschke, V. M., Ryden, M., & Langin, D. (2021). In vitro and ex vivo models of adipocytes. *American Journal of Physiology - Cell Physiology*, *320*(5), C822–C841. https://doi.org/10.1152/ajpcell.00519.2020

Fish, KN. (2022). Total Internal Reflection Fluorescence (TIRF) Microscopy. *Current Protocols*, *2*(8). https://doi.org/10.1002/cpz1.517

Frayn KN. (2002). Adipose tissue as a buffer for daily lipid flux Frayn . *Diabetologia*, *45*(9), 1201–1220.

Gehl J. (2003). Electroporation theory and methods, perspectives for drug delivery, gene therapy and research. *Acta Physiol Scand*, *177*(4), 437–447.

Gliemann, J., Rees, W., & Foley, J. (1984). The fate of labelled glucose molecules in the rat adipocyte. Dependence on glucose concentration. *Biochim Biophys Acta.*, *804*(1), 68–76. 10.1016/0167-4889(84)90100-9. PMID: 6372867.

Guilherme, A., Virbasius, J. V., Puri, V., & Czech, M. P. (2008). Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. In *Nature Reviews Molecular Cell Biology* (Vol. 9, Issue 5, pp. 367–377). https://doi.org/10.1038/nrm2391

Gupta, O. T., & Gupta, R. K. (2024). The Expanding Problem of Regional Adiposity: Revisiting a 1985 Diabetes Classic by Ohlson et al. *Diabetes*, *73*(5), 649–652. https://doi.org/10.2337/dbi24-0021

Gustafson, B., Hedjazifar, S., Gogg, S., Hammarstedt, A., & Smith, U. (2015). Insulin resistance and impaired adipogenesis. *Trends in Endocrinology & Metabolism*, *26*(4), 193–200. https://doi.org/10.1016/j.tem.2015.01.006

Herman, M. A., Peroni, O. D., Villoria, J., Schön, M. R., Abumrad, N. A., Blüher, M., Klein, S., & Kahn, B. B. (2012). A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. *Nature*, *484*(7394), 333–338. https://doi.org/10.1038/nature10986

Heydemann A. (2016). An Overview of Murine High Fat Diet as a Model for Type 2 Diabetes Mellitus. *J Diabetes Res*. 10.1155/2016/2902351.

Hruby, A., & Hu, F. B. (2015). The Epidemiology of Obesity: A Big Picture. In *PharmacoEconomics* (Vol. 33, Issue 7, pp. 673–689). Springer International Publishing. https://doi.org/10.1007/s40273-014-0243-x

Iizuka, K., Bruick, R. K., Liang, G., Horton, J. D., Uyeda, K., & McKnight, S. L. (2004). *Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis*. www.pnas.orgcgidoi10.1073pnas.0401516101

Inoue, N., Yahagi, N., Yamamoto, T., Ishikawa, M., Watanabe, K., Matsuzaka, T., Nakagawa, Y., Takeuchi, Y., Kobayashi, K., Takahashi, A., Suzuki, H., Hasty, A. H., Toyoshima, H., Yamada, N., & Shimano, H. (2008). Cyclin-dependent kinase inhibitor, p21WAF1/CIP1, is involved in adipocyte differentiation and hypertrophy, linking to obesity, and insulin resistance. *Journal of Biological Chemistry*, *283*(30), 21220–21229. https://doi.org/10.1074/jbc.M801824200

Jian Sun, X., Crimmins, D. L., Myers, M. G., Miralpeix, M., & White, M. F. (1993). Pleiotropic Insulin Signals Are Engaged by Multisite Phosphorylation of IRS-1. In *MOLECULAR AND CELLULAR BIOLOGY* (Vol. 13, Issue 12).

Kahle, M., Horsch, M., Fridrich, B., Seelig, A., Schultheiß, J., Leonhardt, J., Irmler, M., Beckers, J., Rathkolb, B., Wolf, E., Franke, N., Gailus-Durner, V., Fuchs, H., de Angelis, M., & Neschen, S. (2013). Phenotypic comparison of common mouse strains developing high-fat diet-induced hepatosteatosis. *Mol Metab.435*, *2*(4), 435–446.

Kane, S., Sano, H., Liu, S. C. H., Asara, J. M., Lane, W. S., Garner, C. C., & Lienhard, G. E. (2002). A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *Journal of Biological Chemistry*, *277*(25), 22115–22118. https://doi.org/10.1074/jbc.C200198200

Kanzaki, M., Watson, R. T., Hou, J. C., Stamnes, M., Saltiel, A. R., & Pessin, J. E. (2002). Small GTP-binding Protein TC10 Differentially Regulates Two Distinct Populations of Filamentous Actin in 3T3L1 Adipocytes □ V. *Molecular Biology of the Cell*, *13*, 2334–2346. https://doi.org/10.1091/mbc.01-10

Kim, J. I., Park, J., Ji, Y., Jo, K., Han, S. M., Sohn, J. H., Shin, K. C., Han, J. S., Jeon, Y. G., Nahmgoong, H., Han, K. H., Kim, J., Kim, S., Choe, S. S., & Kim, J. B. (2019). *During Adipocyte Remodeling, Lipid Droplet Configurations Regulate Insulin Sensitivity through F-Actin and G-Actin Reorganization*. https://doi.org/10

Kim, J., Michael, M., Previs, S., Peroni, O., Mauvais-Jarvis, F., Neschen, S., Kahn, B., Kahn, C., & Shulman, G. (2000). Redistribution of substrates to adipose tissue promotes obesity in mice with selective insulin resistance in muscle . *J Clin Invest.*, *105*(12), 1791–1797. 10.1172/JCI8305. PMID: 10862794; PMCID: PMC378504.

Koch, H., & Weber, Y. (2019). The glucose transporter type 1 (Glut1) syndromes. *Epilepsy Behav.*, *91*, 90–93. doi: 10.1016/j.yebeh.2018.06.010

Kreppel, F., & Kochanek, S. (2008). Modification of adenovirus gene transfer vectors with synthetic polymers: A scientific review and technical guide. In *Molecular Therapy* (Vol. 16, Issue 1, pp. 16–29). Nature Publishing Group. https://doi.org/10.1038/sj.mt.6300321

Lafontan, M. (2012). Historical perspectives in fat cell biology: the fat cell as a model for the investigation of hormonal and metabolic pathways. *Am J Physiol Cell Physiol*, *302*, 327–359. https://doi.org/10.1152/ajpcell.00168.2011.-For

Lansey, M. N., Walker, N. N., Hargett, S. R., Stevens, J. R., & Keller, S. R. (2012). Deletion of Rab GAP AS160 modifies glucose uptake and GLUT4 translocation in primary skeletal muscles and adipocytes and impairs glucose homeostasis. *Am J Physiol Endocrinol Metab*, *303*, 1273–1286. https://doi.org/10.1152/ajpendo.00316.2012.-Tight

Larance, M., Ramm, G., Stöckli, J., van Dam, E., Winata, S., Wasinger, V., Simpson, F., Graham, M., Junutula, J., Guilhaus, M., & James, D. (2005). Characterization of the Role of the Rab GTPase-activating Protein AS160 in Insulin-regulated GLUT4 Trafficking. *J Biol Chem.*, *280*(45), 37803–37813. 10.1074/jbc.M503897200. Epub 2005 Sep 8. PMID: 16154996.

Lee, M. J., Wu, Y., & Fried, S. K. (2013). Adipose tissue heterogeneity: Implication of depot differences in adipose tissue for obesity complications. In *Molecular Aspects of Medicine* (Vol. 34, Issue 1, pp. 1–11). https://doi.org/10.1016/j.mam.2012.10.001

Leto, D., & Saltiel, A. R. (2012). Regulation of glucose transport by insulin: traffic control of GLUT4. *Nature Reviews Molecular Cell Biology*, *13*(6), 383–396. https://doi.org/10.1038/nrm3351

Li, Y., Periwal, V., Cushman, S. W., & Stenkula, K. G. (2016). Adipose cell hypertrophy precedes the appearance of small adipocytes by 3 days in C57BL/6 mouse upon changing to a high fat diet. *Adipocyte*, *5*(1), 81–87. https://doi.org/10.1080/21623945.2015.1128588

Lim, C. Y., Bi, X., Wu, D., Kim, J. B., Gunning, P. W., Hong, W., & Han, W. (2015). Tropomodulin3 is a novel Akt2 effector regulating insulin-stimulated GLUT4 exocytosis through cortical actin remodeling. *Nature Communications*, *6*. https://doi.org/10.1038/ncomms6951

Lizunov, V. A., Matsumoto, H., Zimmerberg, J., Cushman, S. W., & Frolov, V. A. (2005). Insulin stimulates the halting, tethering, and fusion of mobile GLUT4 vesicles in rat adipose cells. *Journal of Cell Biology*, *169*(3), 481–489. https://doi.org/10.1083/jcb.200412069

Luo, J., Deng, Z. L., Luo, X., Tang, N., Song, W. X., Chen, J., Sharff, K. A., Luu, H. H., Haydon, R. C., Kinzler, K. W., Vogelstein, B., & He, T. C. (2007). A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. *Nature Protocols*, *2*(5), 1236–1247. https://doi.org/10.1038/nprot.2007.135

Martin, O. J., Lee, A., & McGraw, T. E. (2006). GLUT4 distribution between the plasma membrane and the intracellular compartments is maintained by an insulin-modulated bipartite dynamic mechanism. *Journal of Biological Chemistry*, *281*(1), 484–490. https://doi.org/10.1074/jbc.M505944200

McLaughlin, T., Sherman, A., Tsao, P., Gonzalez, O., Yee, G., Lamendola, C., Reaven, G. M., & Cushman, S. W. (2007). Enhanced proportion of small adipose cells in insulin-resistant vs insulin-sensitive obese individuals implicates impaired adipogenesis. *Diabetologia*, *50*(8), 1707–1715. https://doi.org/10.1007/s00125-007-0708-y

Mîinea, C. P., Sano, H., Kane, S., Sano, E., Fukuda, M., Peränen, J., Lane, W. S., & Lienhard, G. E. (2005). AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. *Biochemical Journal*, *391*(1), 87–93. https://doi.org/10.1042/BJ20050887

Muretta, J., Romenskaia, I., Cassiday, P., & Mastick, C. (2007). Expression of a synapsinsIIb site 1 phosphorylation mutant in 3t3-L1 adipocytes inhibits basal intracellular retention of Glut4. *Journal of Cell Science*, *120*(7), 1168–1177. https://doi.org/10.1242/jcs.03413

Nagamatsu, S., Nakamichi, Y., Ohara-Imaizumi, M., Ozawa, S., Katahira, H., Watanabe, T., & Ishida, H. (2001). Adenovirus-mediated preproinsulin gene transfer into adipose tissues ameliorates hyperglycemia in obese diabetic KKAy mice. *FEBS Letters*, *509*(1), 106–110. https://doi.org/10.1016/S0014-5793(01)03146-5

Okunogbe, A., Nugent, R., Spencer, G., Powis, J., Ralston, J., & Wilding, J. (2022). Economic impacts of overweight and obesity: current and future estimates for 161 countries. *BMJ Global Health*, *7*(9). https://doi.org/10.1136/bmjgh-2022-009773

Potter H. (1988). Electroporation in biologymethods, applications, and instrumentation. *Anal Biochem*, *174*(2), 361–373.

Poulter, N., Pitkeathly, W., Smith, P., & Rappoport, J. (2015). The physical basis of total internal reflection fluorescence (TIRF) microscopy and its cellular applications. *Methods Mol Biol.*, *1251*, 1–23.

Quon, M., Guerre-Millo, M., Zarnowski, M., Butte, A., Em, M., Cushman, S., & Taylor, S. (1994). Tyrosine kinase-deficient mutant human insulin receptors (Met1153-->Ile) overexpressed in transfected rat adipose cells fail to mediate translocation of epitope-tagged GLUT4. *Proc Natl Acad Sci U S A.* , *91*(12), 5587–5591. 10.1073/pnas.91.12.5587. PMID: 8202531; PMCID: PMC44041.

Rao, T., Nawara, T., & Mattheyses, A. (2022). Live-Cell Total Internal Reflection Fluorescence (TIRF) Microscopy to Investigate Protein Internalization Dynamics. *Methods Mol Biol.* , *2438*, 45–48.

Rauschhuber, C., Noske, N., & Ehrhardt, A. (2012). New insights into stability of recombinant adenovirus vector genomes in mammalian cells. *Eur J Cell Biol*, *91*(1), 2–9.

Rosen ED, S. BM. (2000). Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol.*, *16*, 145–171. 10.1146/annurev.cellbio.16.1.145. PMID: 11031233.

Rosen, M., Grant, P., & Dallon, J. (2021). Mean square displacement for a discrete centroid model of cell motion. *PLoS One*, *16*(12). 0.1371/journal.pone.0261021. PMID: 34928985; PMCID: PMC8687545.

Ruan, H., & Dong, L. Q. (2016). Adiponectin signaling and function in insulin target tissues. In *Journal of Molecular Cell Biology* (Vol. 8, Issue 2, pp. 101–109). Oxford University Press. https://doi.org/10.1093/jmcb/mjw014

Rudich, A., Konrad, D., Török, D., Ben-Romano, R., Huang, C., Niu, W., Garg, R. R., Wijesekara, N., Germinario, R. J., Bilan, P. J., & Klip, A. (2003). Indinavir uncovers different contributions of GLUT4 and GLUT1 towards glucose uptake in muscle and fat cells and tissues. *Diabetologia*, *46*(5), 649–658. https://doi.org/10.1007/s00125-003-1080-1

Sadowski, H. B., Wheeler, T. T., & Young, D. A. (1992). Gene expression during 3T3-L1 adipocyte differentiation: Characterization of initial responses to the inducing agents and changes during commitment to differentiation. *Journal of Biological Chemistry*, *267*(7), 4722–4731. https://doi.org/10.1016/s0021-9258(18)42892-x

Sano, H., Kane, S., Sano, E., Mîinea, C., Asara, J., Lane, W., Garner, C., & Lienhard, G. (2003). Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *Journal of Biological Chemistry*, *278*(17), 14599–14602. https://doi.org/10.1074/jbc.C300063200

Shepherd, P., & Kahn, B. (1999). Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus. *N Engl J Med.* , *341*(4), 248–257.

Simpson, I. A., Dwyer, D., Malide, D., Moley, K. H., Travis, A., Vannucci, S. J., & Simpson, I. A. (2008). The facilitative glucose transporter GLUT3: 20 years of distinction. *Am J Physiol Endocrinol Metab*, *295*, 242–253. https://doi.org/10.1152/ajpendo.90388.2008.-Glucose

Song, Z., Xiaoli, A. M., & Yang, F. (2018). Regulation and metabolic significance of De Novo lipogenesis in adipose tissues. In *Nutrients* (Vol. 10, Issue 10). MDPI AG. https://doi.org/10.3390/nu10101383

Speakman, J. R. (2005). Body size, energy metabolism and lifespan. In *Journal of Experimental Biology* (Vol. 208, Issue 9, pp. 1717–1730). https://doi.org/10.1242/jeb.01556

Stall, R., Ramos, J., Kent Fulcher, F., & Patel, Y. M. (2014). Regulation of myosin IIA and filamentous actin during insulin-stimulated glucose uptake in 3T3-L1 adipocytes. *Experimental Cell Research*, *322*(1), 81–88. https://doi.org/10.1016/j.yexcr.2013.12.011

Stenkula, K. G., Lizunov, V. A., Cushman, S. W., & Zimmerberg, J. (2010). Insulin controls the spatial distribution of GLUT4 on the cell surface through regulation of its postfusion dispersal. *Cell Metabolism*, *12*(3), 250–259. https://doi.org/10.1016/j.cmet.2010.08.005

Thorens, B. (2015). GLUT2, glucose sensing and glucose homeostasis. In *Diabetologia* (Vol. 58, Issue 2, pp. 221–232). Springer Verlag. https://doi.org/10.1007/s00125-014-3451-1

Thurmond, D. C., Gonelle-Gispert, C., Furukawa, M., Halban, P. A., & Pessin, J. E. (2003). Glucose-stimulated insulin secretion is coupled to the interaction of actin with the t-SNARE (target membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein) complex. *Molecular Endocrinology*, *17*(4), 732–742. https://doi.org/10.1210/me.2002-0333

Vogel, A., & Pollack, R. (1973). Isolation and characterization of revertant cell lines. IV. Direct selection of serum-revertant sublines of SV40-transformed 3T3 mouse cells Vogel. *J Cell Physiol*, *82*(2), 189–198.

Wang, H. Y., Ducommun, S., Quan, C., Xie, B., Li, M., Wasserman, D. H., Sakamoto, K., Mackintosh, C., & Chen, S. (2013). AS160 deficiency causes whole-body insulin resistance via composite effects in multiple tissues. *Biochemical Journal*, *449*(2), 479–489. https://doi.org/10.1042/BJ20120702

Wasserstrom, S., Morén, B., & Stenkula, K. (2018). Total Internal Reflection Fluorescence Microscopy to Study GLUT4 Trafficking. *Methods Mol Biol.* , *1713*, 151–159.

Williams, A. S., Kang, L., & Wasserman, D. H. (2015). The extracellular matrix and insulin resistance. In *Trends in Endocrinology and Metabolism* (Vol. 26, Issue 7, pp. 357–366). Elsevier Inc. https://doi.org/10.1016/j.tem.2015.05.006

Yadav, H., Quijano, C., Kamaraju, A. K., Gavrilova, O., Malek, R., Chen, W., Zerfas, P., Zhigang, D., Wright, E. C., Stuelten, C., Sun, P., Lonning, S., Skarulis, M., Sumner, A. E., Finkel, T., & Rane, S. G. (2011). Protection from obesity and diabetes by blockade of TGF-β/Smad3 signaling. *Cell Metabolism*, *14*(1), 67–79. https://doi.org/10.1016/j.cmet.2011.04.013

Yamada, T., Katagiri, H., Ishigaki, Y., Ogihara, T., Imai, J., Uno, K., Hasegawa, Y., Gao, J., Ishihara, H., Niijima, A., Mano, H., Aburatani, H., Asano, T., & Oka, Y. (2006). Signals from intra-abdominal fat modulate insulin and leptin sensitivity through different mechanisms: Neuronal involvement in food-intake regulation. *Cell Metabolism*, *3*(3), 223–229. https://doi.org/10.1016/j.cmet.2006.02.001

Yang, A., & Mottillo, E. P. (2020). Adipocyte lipolysis: From molecular mechanisms of regulation to disease and therapeutics. In *Biochemical Journal* (Vol. 477, Issue 5, pp. 985–1008). Portland Press Ltd. https://doi.org/10.1042/BCJ20190468

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