January 2019

Insulin And Non-Insulin Dependent Glut4 Trafficking: Regulation By The Tug Protein

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A Thesis Submitted to

the Yale University School of Medicine

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Medicine

by

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2019

Abstract

The body tightly regulates glucose production and disposal despite changing metabolic demands, including large post-prandial and fasting fluctuations. Specifically, under the action of insulin, muscle contraction, ischemia, and poor nutrient availability, cells increase the amount of the glucose transporter type 4 (GLUT4) at the plasma membrane by mobilizing a sequestered pool of transporters. In this work, we demonstrate that the TUG (tether containing a UBX domain for GLUT4) protein mediates both insulin-dependent and insulin-independent pathways to increase GLUT4 at the plasma membrane. In mice fed a high fat diet to induce insulin resistance, the regulation of the endoproteolytic cleavage of the TUG protein was disrupted. We also present evidence that helps to identify the key protease, Usp25m, that cleaves the tethering protein TUG in both an insulin-dependent and insulin-independent manner, releasing GLUT4 from its storage location in the basal state to the plasma membrane in an activated state. Finally, our results also suggest that in the adipocytes and myocytes, activated AMPK leads to cleavage of the TUG protein.

Acknowledgements

I would like to thank Dr. Estifanos Habtemichael, who patiently taught me the techniques necessary to work in a cell biology laboratory at the beginning of medical school. His constant guidance and feedback were invaluable to the work that led to this thesis. I would also like to thank Don Li, who helped me adapt and optimize my planned projects. His mentoring both in research and in medical training has been a central part of my training as a physician and as a scientist. Finally, I would like to thank my advisor, Dr. Jonathan Bogan, whose constant enthusiasm for science, optimism, and support made working in his lab the highlight of my time in medical school.

Table of contents

1. Introduction

1.1. Insulin resistance

The human body, in terms of its nutritional requirements, is optimized for a time when food was scarce and unpredictable (1). However, over the last half-century in the developed world and now increasingly in developing countries, dense caloric foods have become easily available. Obesity, as well as comorbid conditions like non-alcoholic fatty liver disease (NAFLD) and atherosclerosis, are on the rise. Type 2 diabetes in particular is implicated in significant and growing morbidity and mortality in the United States and worldwide (2). The burdens of this disease include kidney failure, retinopathy, and neuropathy (3). While type 2 diabetes has been a major health problem in developed countries for decades, the populations with the largest rates of increase in the disease are in Asia and the Indian subcontinent. In these countries, the number of people living with type 2 diabetes is projected to increase by over 75% by the year 2034.

The rising number of cases of type 2 diabetes has been apparent in the United States for decades. The prevalence of diabetes in the United States increased from 0.9% in 1958 to 4.4% in 2000, and 90%-95% of these diagnoses were type 2 diabetes. Notably, the increase in cases over this forty year period was not uniform across age groups: in the 18-29 year-old group, the increase in diabetes diagnoses went up by 40%, while in persons aged 30-39, the increase was 95%. For older patients aged 40-49, the number of diagnoses rose by 83%, and in patients aged 50-59, 49%. The rates were slowest to rise in the oldest age groups; in patients 60-69, diagnoses rose by 40%, while in patients over 70, 33% (4).

Over the last few decades, both epidemiological and laboratory studies have shed light on the pathophysiology of type 2 diabetes. At a basic level, insulin resistance leads to the disease, and beta-cell dysfunction follows (5).

However, it remains unclear if hyperinsulinemia is the primary cause of insulin resistance, or if it is entirely secondary (6). The former model is supported by evidence that fasting hyperinsulinemia may develop before an increase in postprandial blood glucose, causing the release of insulin from beta-cells (7). The latter model, in which insulin resistance precedes hyperinsulinemia, is perhaps more widely accepted. Patients who are insulin resistant because of known mutations in their insulin-signaling pathways provide some of the strongest evidence for this model. In these patients, the primary lesion is resistance; observable effects, including hyperinsulinemia, are therefore concluded to be secondary phenomena (8). In this model, with the effects of insulin blunted, the beta cells of the pancreas compensate by increasing the production of insulin.

In 1963, Randle and others published a paper connecting obesity to insulin resistance, arguing that glucose oxidation was impaired by the presence of high levels of fatty acid (9). Today, overwhelming epidemiological data, along with mouse and human data, illustrate that caloric imbalance is a key causative factor in the development of insulin resistance, and thus a risk factor for the development of type 2 diabetes (7). However the specific mechanistic connections between obesity and insulin resistance remain somewhat unclear.

Notably, one of the many sequela of the obesity epidemic has been a drastic rise in the prevalence of NAFLD. Today it is a major cause of liver disorder in the Western world (10). Fat deposits in the liver are strongly associated with type 2 diabetes – more

than 90% of obese patients with type 2 diabetes have NAFLD – and insulin resistance is a common feature of both NAFLD and obesity (10). Two recent studies in particular (12, 13) have advanced the understanding of the mechanisms that link deposits of fat in the liver and resistance to insulin. Plasma-free fatty acids were observed at higher levels in patients who were obese and had type 2 diabetes, and further work established an inverse relationship between fasting plasma-free fatty acid concentrations and insulin sensitivity, more proximally linking plasma-free fatty acids with insulin resistance (11, 12). Subsequent studies, using ^{1}H NMR (proton nuclear magnetic resonance) and muscle biopsy, have more directly demonstrated the strong link between high concentrations of intramyocellular triglycerides and insulin resistance (12). This can lead to NAFLD by causing a shift in the distribution of energy substrates, so that they accumulate in the liver and are stored as fat.

While these results might seem consistent with the Randle hypothesis – implicating fatty acids as causal to insulin resistance – more recent work suggested an important mechanistic difference. Under the Randle hypothesis, which was developed using cardiac and diaphragm muscle, increased citrate concentrations affect phosphofructokinase. However, recent data support the idea that fatty acids interfere with an early step in the signaling cascade that ultimately leads GLUT4 to translocate to the cell surface (9, 12). Diacylglycerides were later identified as a causal factor in hepatic insulin resistance. In hepatocytes, a novel protein kinase C isoform, PKCε, was found to be translocated to the plasma membrane, inhibiting the activity of the intracellular kinase domain of the insulin receptor (13, 14). In myocytes, a different novel protein kinase C, PKCθ, has been identified (15). The mechanism for resistance to insulin signaling in

hepatocytes caused by diacylglycerides was further explained in 2016. The phosphorylation site (Thr1160) was identified as a substrate of PKCε in the kinase activation loop of the insulin receptor. In vitro studies showed that a mutation from threonine to glutamic acid (T1160E), which can mimic phosphorylation, caused impaired insulin receptor signaling, but a mutation to alanine (T1160A), which resists phosphorylation, did not show inhibition. Furthermore, in mice, mutation from threonine to alanine on Thr1150 (the homologous residue in mice) conferred protection in the insulin signaling pathway in mice that were fed a high-fat diet to induce hepatic insulin resistance (16). Further experiments showed crosstalk between PKCε and the kinase p70S6k (17). This work clearly demonstrated that insulin signaling in the liver is deranged due to accumulation of lipids.

However, changes in insulin signaling do not fully explain the phenomenon of insulin resistance. The downstream consequence of insulin signaling is GLUT4 translocation to the plasma membrane, and changes to the abundance and distribution of GLUT4 have been demonstrated in muscle and adipose tissue. Garvey and others studied muscle and adipose tissues of humans during fasting, and compared patients with type 2 diabetes to healthy controls. Notably, compared to the controls, GLUT4 targeting was altered in the type 2 diabetic group even in the fasting state, when insulin signaling is minimal. In adipose, GLUT4 was depleted in all membrane sub-fractions, and the co-trafficking protein IRAP was similarly altered in its basal distribution among intracellular membranes in the fasting state (18). These results suggest that alterations in membrane trafficking may contribute to insulin resistance, independent of alterations in insulin signaling pathways.

1.2. Macronutrient contributions to obesity

The previous section addressed the mechanistic connections between obesity and insulin resistance, but the contributing factors to obesity are also important to consider. In 2017, the Endocrine Society published a scientific review, making the case that caloric imbalance has been a dominant factor in the rise of obesity. The review proposed that irrespective of the macronutrient balance, the calorie amount is the determining factor in weight gain; in simple terms, "a calorie is a calorie" (19).

A prominent opposing view, often called the carbohydrate-insulin model, holds that the macronutrient content of ingested calories is important to determine weight and obesity. Changes in dietary quality in the last 50 years may have caused hormonal responses that shift calories towards deposition of fat (20). Under the carbohydrate-insulin model, if calories are stored, then the energy content of blood is reduced, which causes hunger and subsequent overeating; in other words, a high-carbohydrate diet causes postprandial hyperinsulinemia and this promotes deposition of fat (20). As Ludwig and Ebbeling note, the carbohydrate-insulin model does not violate the First Law of Thermodynamics (conservation of energy). This model sees overeating as a consequence of increased fat stores, and not the primary cause (20). The details of the supporting evidence for the carbohydrate-insulin model, including considerable human, animal, and cell-culture research, are beyond the scope of this thesis (20), but the most recent supporting study was a randomized human trial, published in late 2018 (21).

Advocates for the conventional model ("a calorie is a calorie") argue that there are key flaws in the carbohydrate-insulin model. Specifically, in the carbohydrate-insulin model, because fuels are being stored under the action of insulin, the level of circulating

fuels in the blood is reduced, leading to hunger and thus increased food intake. But as Hall and others argue, obese individuals have normal or even elevated levels of circulating fuels, including free fatty acids and glucose, and the adipose of obese patients actually releases more total free fatty acids and glycerol than individuals of normal weight. Furthermore, diets with varying degrees of glycemic index and load do not lead to a significant difference in hunger, either acutely or longer term (22, 23). Finally, Hall and others note that patients on strict diets with lower glycemic indices had lower levels of insulin compared to patients on equal calorie diets with higher glycemic loads, but the patients on diets with lower glycemic loads did not show increased mobilization of free fatty acids and oxidation beyond the amount that could be explained by the increased fat intake in their diets. There were not significant differences in weight loss between patients with lower and higher glycemic load diets, and furthermore, decreased insulin levels had no predictive value on weight loss (23, 24).

The macronutrient content required to halt the progression of obesity or lose weight is not only a fundamental question in diabetes research, but a key problem to solve in order to lessen the burden of the associated diseases. While no one study is likely to settle the debate, advocates of the carbohydrate-insulin model recently published the results of a randomized trial in which participants who had lost about 12% of their pre-weight loss body mass on a conventional diet were assigned high and low carbohydrate diets for weight maintenance. The primary outcome was energy expenditure, which was significantly increased in patients assigned the lower carbohydrate diets. The results were most dramatic in patients with the highest pre-weight loss levels of insulin. The patients in the highest third of pre-weight loss insulin levels had an energy expenditure increase of

308 kcals on average on the low carbohydrate diet, compared to patients on the higher carbohydrate diets (25).

1.3. GLUT4 transporters and their regulation by membrane trafficking

The phenomenon of increased movement of glucose down its concentration gradient from the blood to the intracellular space caused by the action of insulin was well documented in pioneering work from the 1970s and 1980s. A major breakthrough was made in 1980, when two groups independently showed that in insulin responsive cells, glucose mobilized a glucose transporting activity from an internal cellular storage location to the plasma membrane (26, 27). Then, in 1981, it was demonstrated that in incubated rat adipocytes, insulin increased glucose transport into the cells, a glucose transporter moved from an intracellular pool to the plasma membrane, and high concentrations of anti-insulin antibodies largely ablated these effects (28). The first biochemical characterization of the factor that facilitates glucose transport was published in 1985 (29). Now termed GLUT1, it is widely expressed, and its location in the cell is not significantly changed by insulin. Further studies characterized a glucose transporter that likely increased in concentration at the plasma membrane, allowing for the diffusion of glucose. Under the action of insulin, transporters would translocate from organelles inside the cell and embed themselves in the plasma membrane. Once embedded in the plasma membrane, glucose would diffuse down its concentration gradient (i.e., ATP-independent transport). Further characterization of the major insulin-sensitive glucose transporter, GLUT4, was described later (30). The GLUT isoform that is responsive to insulin was cloned by five laboratories (31-35). and the gene that encoded the transporter

was termed SLC2A4 (32). The location of the intracellular storage location of GLUT4 is currently an active area of research. Interestingly, in the basal state, GLUT4 is not localized to one compartment or organelle, but two, divided about equally in cultured cells. Thus, the late 1980s marked a key transition in diabetes research. The physiological problem of how insulin increases glucose uptake turned into a cell-biology problem of how insulin signals, and how it causes translocation of the insulin sensitive GLUT isoform, GLUT4, to the plasma membrane (36).

After the role of GLUT4 as a key effector of insulin mediated glucose uptake was recognized, an important challenge remained: describing the signal cascade and mechanism of action that, in the end, leads to GLUT4 being embedded in the plasma membrane (37). It was demonstrated that insulin binds to its receptor, causing dimerization or reorientation of the dimer, and thus activation (38). Once activated, the tyrosine kinase phosphorylates insulin receptor substrates (IRS proteins), in addition to activating phosphatidylinositol-3-kinase (PI3K), and other effector proteins (37, 39).

Downstream, Akt2 (there are 3 isoforms) phosphorylates numerous targets, including AS160 (Akt substrate of 160 kDa) GTPase-activation protein (36, 40). The discovery of AS160 was especially significant because RAB proteins direct vesicle trafficking, so AS160 links signaling and trafficking pathways (36). AS160 is present on GSVs (GLUT4 storage vesicles, discussed in more detail below), and interacts with LRP1 and IRAP (41, 42).

Some of the mechanisms of GLUT4 retention, recycling, and translocation can be gleaned from kinetic data and total internal reflection fluorescence (TIRF) microscopy. The TIRF data was acquired and analyzed long after other methods were used to discover

significant portions of the signaling cascade and retention mechanisms (43). In the basal state, the vast majority of GLUT4 is sequestered intracellularly. One fraction of GLUT4 colocalizes with markers of the recycling endosome (such as the transferrin receptor), while another fraction colocalizes with the Golgi network or endoplasmic reticulum (44).

The dynamics of the GLUT4 transporter have been the subject of much study and controversy. In unstimulated adipocytes and myocytes, the balance of movement to the plasma membrane and to intracellular compartments favors intracellular compartments (45). When insulin (or in the case of myocytes, a contraction or other activating signal) is present, then the balance favors GLUT4 to be inserted into the plasma membrane. This could occur because of a decreased rate of endocytosis or an increased rate of exocytosis. Although the former may occur to a small extent, most data support a model in which the main effect of insulin is to increase GLUT4 at the plasma membrane by increasing the exocytotic arm of the GLUT4 recycling pathway (46).

While it is clear that insulin increases the amount of GLUT4 in the plasma membrane, the mechanism by which GLUT4 is retained and cycled to the plasma membrane remains unclear. One model, advocated by McGraw and others, proposes that, even in the basal state, all GLUT4 in the cell will cycle to the plasma membrane. Another model, advocated by James and others, proposes that in the basal state, some fraction of GLUT4 is sequestered intracellularly, and will not cycle to the plasma membrane unless stimulated by insulin (45). The evidence for each of these models is seemingly quite strong. One notable study showed that all GLUT4 in the cell eventually cycles to the plasma membrane, even in the basal state (47). Evidence that may reconcile these two seemingly contradictory models was published in 2008 (48). In these experiments, re-

plating adipocytes after differentiation caused a much higher proportion of GLUT4 to cycle to the plasma membrane in the basal state. To a large degree, re-plating the cells appeared to disrupt the static retention of GLUT4 in the basal state and cause more GLUT4 to cycle to the plasma membrane, even in the absence of insulin. These observations suggested that the cycling seen in re-plated cells might be an artifact of the disruption.

To allow for the regulation of glucose uptake into fat and muscle cells, GLUT4 glucose transporters are sequestered intracellularly during the basal (low insulin) state. TUG proteins, encoded by the ASPSCR1 gene, play an important role in this process; they serve as anchors that retain GLUT4 in a pool of intracellular "GLUT4 storage vesicles" (GSVs) (49). In addition to GLUT4, GSVs contain insulin-regulated aminopeptidase (IRAP), sortilin, vesicle-associated membrane protein (VAMP2), and low density lipoprotein receptor-related protein 1 (LRP1) (50). The physiologic rationale for having this set of cargos grouped together, all mobilized downstream of insulin signaling, is not clear. For example, the role of IRAP, another cargo and a transmembrane aminopeptidase, involves cleaving several peptide hormones. In a mouse model, vasopressin was shown be a substrate for IRAP, and IRAP-deficient mice had significantly increased levels of vasopressin (51).

TUG was first recognized as a downstream effector of insulin from a functional screen that studied GLUT4 localization (52). Subsequent work aimed to understand the mechanism of its action; while the functional screen identified the TUG protein as having some role in GLUT4 localization, its relative importance in GLUT4 mobilization to the plasma membrane and thus glucose disposal was not known.

A major advance in understanding the importance of TUG came in 2007, with the publication of a study that showed that small interfering RNAs (siRNAs) targeted against the TUG protein mimicked much of the effect of insulin. In cells expressing the siRNAs, GLUT4 translocated from intracellular storage to the plasma membrane, even in the basal (unstimulated) state. This effect could be slightly increased by stimulation with insulin (49). The role of TUG was further illuminated by the discovery that TUG directly binds to an intracellular loop of GLUT4 (49). It appeared that TUG, under the action of insulin, released stored GLUT4 from the Golgi to the plasma membrane. But, how, specifically, did insulin mediate this effect through TUG? Clarification on this point was made in 2012, when it was discovered that under the action of insulin, the intact TUG protein was cleaved at a specific location, between residues 164 and 165 of the intact 550 residue mouse TUG protein. The result of this cleavage is to divide the 60 kDa intact TUG protein into a 42 kDa C-terminal product and an 18 kDa N-terminal product (53). The current understanding of the role of TUG is that insulin stimulates the endoproteolytic cleavage of TUG to mobilize the GSVs for translocation to the cell surface, so that GLUT4 is inserted into the plasma membrane. Once in the plasma membrane, GLUT4 transports glucose into the cell via facilitated diffusion (53).

To gain further insight into the mechanisms of insulin action, the fusion of individual GSVs in 3T3-L1 adipocytes was monitored in live cells. These experiments required the development of a new approach, because biochemical assays can neither determine the location of GLUT4 at individual trafficking steps nor distinguish between greater abundance of GLUT4 in a compartment due to increased exocytosis or decreased endocytosis (43). The new approach for tracking individual vesicles containing GLUT4

took advantage of TIRF microscopy and a new GSV reporter, VAMP2-pHluorin. The key motivating questions for this work were two-fold: (1) when GLUT4 is inserted into the plasma membrane, did the GLUT4 molecules come from a GSV or an endosome? And (2), what processes could change the relative amounts of GLUT4 from each of these two source compartments at the plasma membrane?

Although GLUT4 trafficking dynamics remain an area of active research, the results of this work strongly suggested that GLUT4 resides in two major compartments, GSVs and endosomes, in cultured 3T3-L1 adipocytes (54, 55). Although distinguishing between these two compartments biochemically based on the different cargo proteins is straightforward, tracking individual fusion events in a live cell is challenging. GSVs and endosomes contain different proteins and also differ greatly in size. GSVs are about 50-70 nm in diameter, while endosomes are much larger, around 100-250 nm in diameter (56). TIRF microscopy enabled the size of individual fusion events to be measured and the moment of docking and fusion to be determined. The experiments suggested that there were two distinct sizes of GLUT4-containing vesicles at fusion: in pre-adipocytes, the size of the vesicles was 153 +/- 42 nm (consistent with the size of endosomes), but in 3T3-L1 adipocytes, the size of the vesicles was just 56 nm +/- 27 nm (consistent with the size of GSVs, which are present in adipocytes but not pre-adipocytes) (43, 56). Finally, in cells with a TUG knockdown, the exocytotic rate of GSVs was similar to the exocytotic rate of control 3T3-L1 adipocytes during insulin stimulation. This last observation supports a model in which TUG retains GLUT4 in the basal state and is cleaved upon insulin stimulation. A model of TUGs action is summarized in Fig. 1 (36).

While these TIRF studies indicate that TUG is important for trafficking in insulin responsive cells, the role of TUG in protein trafficking may extend to other cell types as well. For example, in HeLa cells, TUG was found localized in the endoplasmic reticulum-to-Golgi intermediate compartment (ERGIC) and the endoplasmic reticulum exit sites (ERES). The vast majority of TUG was bound to p97/VCP, which is a hexamaric ATPase that is important in membrane fusion and proteolysis. In these HeLa cells, TUG caused disassembly of hexamers into monomers, controlling their functional status. Furthermore, a knockdown of TUG disrupted the Golgi. These results suggest that TUG may have an important role in the early secretory pathway in multiple cell types, not only in adipocytes and myocytes (57).

Most recently, the mechanism of GSV movement from the Golgi to the plasma membrane has been described in detail. This discovery was underpinned by the observation that an antibody directed against the 18 kDa N-terminal product of TUG cleavage also detected a protein of approximately 130 kDa. This product was not seen after knockdown of TUG or without insulin stimulation, suggesting that this is a specific band (49). Analysis of the 18 kDa N-terminal cleavage product revealed that it contained two ubiquitin-like domains, and ended in a diglycine motif. These characteristics, along with evidence of the 18 kDa covalently attaching to another protein to form the 130 kDa product, led investigators to hypothesize that the N-terminal product was acting in a similar manner to a ubiquitin-like modifier. As a result, it was named TUGUL (TUG Ubiquitin-Like).

Ubiquitin is best known for its role in covalently attaching to numerous proteins and targeting them for destruction in the proteasome. Ubiquitination is similar to

phosphorylation and acetylation in that they are post-translational modifications that change protein function. In the context of a cell's acute response to increased insulin concentration and translocation of GLUT4, the short timescale of a post-translational modification is important, as changes in gene transcription and translation are too slow to either dispose of glucose in a post-prandial setting, or to allow a cell to increase glucose transport when it has an acute need for more energy.

Ubiquitin is one of a family of similar modifiers termed ubiquitin-liked modifiers (UBLs). While the short time course of action is similar to small molecule modifiers, there are a number of differences (58). Ubiquitin is a 76-residue protein, highly conserved among eukaryotes but absent from bacteria and archaea (59). Interestingly, while UBLs do not always share high sequence similarly, they have a similar three-dimensional structure (59, 60). The structure of UBLs, because of their size and diversity, exhibit functions that differ from small molecules. For example, UBLs can alter protein conformation or protein-protein interactions (58). In general, UBLs are synthesized as non-functional precursors. Next, the protein is modified so that there is a Gly-Gly motif at the C-terminus, the site of attachment to the target. This step is carried out by a group of enzymes termed de-ubiquitinating enzymes (DUBs). An ubiquitin–activation enzyme, termed E1, then adenylates the modified C-terminus. The ubiquitin molecule is passed to a cysteinyl group on the second ubiquitin-conjugation enzyme, E2. The final step is accomplished by a ubiquitin-protein ligase, called E3 (58). There numerous types of E2 and E3 enzymes, not just one form. As the list of UBLs grows, it has been challenging to identify which features are unique, and which are common to all UBLs. E3 appears to not always be required; among other examples, small ubiquitin-related modifier (SUMO)

appears to not always require E3 activity (58). Of possible relevance to GLUT4 trafficking is the observation that membrane-protein-sorting factors are mono-ubiquinated. Ubiquitin thus appears to serve as an important modification to direct many types of cell traffic to different compartments, depending on cytosolic conditions (61). The identification of TUGUL as a novel ubiquitin-like protein modifier implied that a DUB, as well as E1, E2, and E3-like enzymes, may be involved in insulin action, and that these proteins may mediate TUGUL generation and covalent modification of a target protein to promote glucose uptake (62).

1.4. Thyroid hormone agonists

Using thyroid hormone mimetics to agonize receptors has the potential to increase lipid metabolism, decrease low-density lipoprotein (LDL), increase energy expenditure, and increase thermogenesis (63). Thyroid hormones exert their effects by action at one of three nuclear receptors: TR α , TR β 1, and TR β 2, that are distributed in a tissue specific fashion (63). Naturally occurring thyroid hormone acts equally on all three of these receptors. An agonist that has desirable metabolic effects could also agonize thyroid receptors in all tissues, causing ill effects such as tachyarrhythmias, osteoporosis, agitation, or psychosis (63). One strategy to exploit the positive metabolic benefits of thyroid hormone actions while negating the harmful effects is to develop synthetic analogs that agonize only one receptor. The TR β 1 predominates in the liver, so this is an attractive target (63). A recent study that tested two thyroid hormone TR β 1 agonists showed that while fat accumulation in liver was markedly decreased in the presence of TR β 1, sensitivity to insulin also decreased. One of these TR β 1 agonists, KB-2115,

caused a notable decrease in GLUT4 and TUG, yet sortilin, a marker of GSVs, was not reduced (63). One explanation for the decreased sensitivity to insulin, as well as decreased TUG and GLUT4 in the presence of KB-2115, could be that even though GSVs are formed, they are not sequestered in an insulin-responsive pool as is normal (63).

1.5. Exercise-induced glucose uptake in muscle and the role of AMPK

In addition to insulin, exercise also increases GLUT4 in the plasma membrane. During exercise, there is a large increase in the demand for ATP. Early in the exercise period, stored glycogen is the main source of energy. But, as the length of the exercise period continues and glycogen stores become exhausted, blood glucose accounts for around 35% of oxidative metabolism and nearly all of the muscle carbohydrate metabolism (64). Regular exercise improves glycemic control in patients with type 2 diabetes (65). In some ways, this is intuitive: acute increased demand for glucose in muscle cells reduces the concentration of glucose in the serum. But type 2 diabetes is marked by insensitivity to insulin, which among its many functions, is crucial to disposal of serum glucose in muscle in the post-prandial period. Thus it is actually an important finding that even in patients who are resistant to insulin's effect on glucose disposal, exercise-induced glucose disposal mechanisms are still intact. The results of Martin and others – suggesting that the main cause of reduced blood glucose after exercise in patients with type 2 diabetes is increased muscle uptake of glucose instead of reduced glucose output by the liver – further confirmed a key difference between insulin and exercise-induced glucose uptake in muscle (64, 66). Together, these results could lead to new

therapeutic strategies, as at least some mechanisms for glucose uptake are still intact in patients with type 2 diabetes.

Ruling out decreased hepatic glucose production as the cause of decreased serum glucose after exercise does not identify the rate-limiting step at the level of the muscle tissue. In vivo, the rate-limiting step could be glucose delivery to the skeletal muscle, glucose transport across the plasma membrane, or flux through intracellular metabolism (64). At first glance, the most likely candidate might seem to be increased glucose delivery, as blood flow can increase up to 20-fold (at least partly due to IRAP, a GSV cargo that degrades vasopressin) during exercise (64, 67). In fact, all three of these elements probably play some role in glucose disposal, but compelling evidence points towards transport across the plasma membrane as the key regulatory step (68). Two aspects of this research have important implications for GLUT4 trafficking to the plasma membrane: (1) contraction-stimulated uptake is normal in subjects resistant to insulin, and (2) glucose uptake (and hence GLUT4 trafficking to the plasma membrane) is a crucial regulatory step in exercise. Two intriguing questions, then, are what steps in the signaling pathway are common to both exercise and insulin-stimulated glucose uptake, and where is the derangement in insulin resistance such that insulin does not cause normal GLUT4 trafficking, but exercise does? These are outstanding challenges for the field, but we address some commonalities between the signaling pathways of exercise-induced and insulin-induced GLUT4 translocation in the results section.

Upstream in the signaling pathway, AMPK (5' adenosine monophosphate-activated protein kinase) acts as important energy sensor in exercising muscles. It is activated in low energy states, causing the cell to decrease ATP consumption and

increase ATP production. In other words, it acts as a metabolic switch, shifting the body from an anabolic state to a catabolic state (69). The degree of anabolic or catabolic status is primarily determined by the ratio of AMP and ADP to ATP. Initially, AMP was thought to be the primary activator of AMPK, but more recent studies have suggested that ADP is in fact a more important regulator (70). Along with its major upstream kinase, liver kinase B1 (LKB1), AMPK is the most widely-studied protein implicated in skeletal muscle glucose transport in response to exercise (71). It is a heterotrimer of three subunits: a catalytic susubunit, as well as β and γ subunits. There are several isoforms of the subunits that are expressed in different cell types. The critical serine-threonine kinase domain is in the α ssubunit and the activating residue is Thr^{172} (69). Mutations to the γ subunit have been implicated in cardiac disease. The gene (PRKAG2), which encodes the $\gamma 2$ subunit, is linked to familial hypertrophic cardiomyopathy (HCM) associated with Wolff-Parkinson-White syndrome. While the more common forms of HCM involve sarcomeres, PRKAG2 syndrome is associated with accumulation of glycogen in cardiac myocytes and does not involve disarray of the fibers of the cardiac myocytes (69, 72).

In the heart, numerous factors can increase AMPK activity, including physiological stress, hormones, or drugs, including metformin, phenformin, thiazolidinediones, salicylates, 5-aminoimidazole-4-carboxamide riboside (AICAR), and A-769662 (69). Interestingly, insulin actually decreases activation of AMPK through the Akt pathway (69, 73). In addition to the short-term effects of the activation of AMPK, the $\alpha 2$ isoform has a nuclear localization signal. Translocation to the nucleus appears to require activation by phosphorylation at Thr^{172} (69, 74).

AMPK is required for insulin-independent GLUT4 translocation during states of energy stress, such as cardiac ischemia or skeletal muscle contraction (75-77). In 1999, it was found that AMPK mediates GLUT4 translocation independently of the PI3K pathway, an important insight for cardiac physiology (78). AMPK activation also enhances the sensitivity of muscle cells to insulin-dependent GLUT4 translocation and glucose uptake, and may mediate the enhanced insulin sensitivity that occurs after exercise (40, 79). Increased disposal of glucose in the acute post-exercise period (about 2-3 hours) is insulin-independent. But after this acute effect wears off, enhanced muscle and whole body insulin sensitivity can persist for up to 48 hours (40). AMPK can be activated by high doses of metformin (80). Phenformin, a similar compound to metformin, poisons the mitochondria, interfering with complex I of the electron transport chain, slowing the production of ATP, and thus decreasing the ratio of ATP to AMP (81).

The first known drug that was shown to activate AMPK in cells was AICAR (82). AICAR, an adenosine analog, is taken up by cells via adenosine transporters and phosphorylated by adenosine kinase, which generates an AMP mimic, AICAR monophosphate (ZMP) (83). In a screen of compounds for activators of AMPK, a drug named A-592017 was a promising candidate after the initial screen. After optimization, an even more potent activator, A-769662, was developed (82, 84). One rationale for this kind of search for a more potent and direct activator of AMPK is to gain the therapeutic effects seen with the biguanides while reducing or eliminating the side effects which may stem from the biguanides inhibiting the respiratory chain, rather than from the direct activation of AMPK (82). A-769662 is thought to work by directly activating AMPK through two mechanisms: allosteric activation and inhibition of dephosphyorlylation (82).

The therapeutic effect of metformin to lower the blood glucose level of patients with type 2 diabetes is due to the action of metformin on hepatic glucose production, although metformin has also been proposed to increase skeletal muscle glucose uptake mediated through its action on AMPK (85). The mechanism for decreased glucose production by the liver is metformin's targeting of a mitochondrial enzyme that controls the cellular redox state, as well as ATP production. Thus, activation of AMPK may not be necessary for this reduction (86-88). The specific enzyme affected by metformin was recently discovered, after the observation that animals treated with biguanides had increased levels of lactate. Metformin non-competitively inhibits the redox shuttle enzyme mitochondrial glycerophosphate dehydrogenase. This process reduces the conversion of lactate and glycerol to glucose, and thus decreases hepatic gluconeogenesis (86).

Recent results showed that after induced cardiac ischemia, well-known to activate AMPK, with subsequent reperfusion (I/R), TUG is cleaved, at least partly explaining increased GLUT4 and other GSV cargos at the plasma membrane after reperfusion (89).

In addition to AMPK, calmodulin signaling and calmodulin-dependent protein kinases (CaMKs) have been implicated as vital components of exercise-stimulated skeletal muscle glucose uptake (90). Whether this path is independent of AMPK is controversial. In support of a model in which CaMKs are independent of AMPK, studies have shown that the incubation of rat skeletal muscle with KN-93, a Ca^{2+}Calmodulin inhibitor, decreased the uptake of glucose after contraction (71, 91). These studies also demonstrated significant inhibition of exercise-induced Cam-KII phosphorylation without AMPK inhibition.

On the other hand, a number of studies support a model in which AMPK activation is required for contraction-mediated glucose uptake in muscle cells. These studies were conducted in incubated soleus muscles from wildtype mice and mice with a muscle specific-kinase-dead (KD) AMPK, $\alpha 1$ AMPK knockout, or $\alpha 2$ AMPK knockout. Glucose uptake was monitored using 2-deoxyglucose. Muscle contraction increased the activity of the $\alpha 1$ subunit of AMPK mice, but not the $\alpha 2$ subunit of AMPK mice (92). These studies also showed that when CaMK signaling was inhibited with KN-93, contraction-induced skeletal muscle glucose uptake through an AMPK-dependent signaling pathway was also inhibited (71, 92).

1.6. Mouse model of type 2 diabetes mellitus

To effectively study type 2 diabetes, a model that reproduces the phenotype is required. The two major methods for producing a mouse model of type 2 diabetes are genetic manipulation and diet induction. Mice fed a high fat diet (HFD) are well known to develop insulin resistance (93). A major advantage of using a high fat diet to induce type 2 diabetes phenotypes is the large number of genes identified in genome-wide association studies (GWAS). Over 50 genes have been implicated in humans, so one mouse model cannot replicate this diversity (93). While mice with genetic deficits, such as the ob/ob mouse, have some utility in studying type 2 diabetes mellitus, HFD-fed mice exhibit a more physiologic model than that of monogenetic mutations.

2. Statement of purpose, specific hypothesis, and specific aims

This thesis encompasses three distinct parts that together seek to elucidate the mechanism of GLUT4 translocation to the plasma membrane and the derangements in this process in patients with insulin resistance.

Specifically, we hypothesize that the protease Usp25m (ubiquitin specific peptidase 25) interacts with TUG. This work is part of a larger project aimed at showing that Usp25m is responsible for insulin-dependent endoproteolytic cleavage of the TUG protein, and as discussed in detail below, Usp25m is also likely responsible for insulin-independent (AMPK-mediated) TUG cleavage. The project was motivated by preliminary data that suggested that Usp25m might be a candidate protease to cleave the TUG protein. In our model, Usp25m directly cleaves the 60 kDa intact TUG protein into a 42 kDa C-terminal product and an 18 kDa N-terminal product. As a first step towards confirming the identity of the TUG protease, it is necessary to show that Usp25 associates with TUG, using coimmunoprecipitation.

Insulin is not the sole mediator of increased GLUT4 at the plasma membrane. We further hypothesize that (1) activated AMPK phosphorylates TUG, and (2) in both an insulin-dependent (as discussed above) and insulin-independent manner, Usp25m is the protease that cleaves TUG, ultimately resulting in GLUT4 mobilization to the cell surface. This proposed mechanism can account for, at least in part, how AMPK activation increases glucose uptake independent of insulin.

Our preliminary data suggested that TUG is a direct target of AMPK-mediated phosphorylation, at least in vitro, and that the specific residue at which AMPK-mediated phosphorylation of TUG occurs is Threonine-57 (T57), which is within the first

ubiquitin-like domain of TUG (94). There is significant evidence that the presence of AMPK increases the amount of GLUT4 embedded in the plasma membrane of fat and muscle cells, but the mechanism for this process is not understood (85). The results of our study will shed light on this mechanism, an important piece of the metabolic response to muscle contraction and cardiac ischemia. If AMPK-mediated TUG cleavage produces the same 42 kDa C-terminal and N-terminal 18 kDa products as insulin-mediated TUG cleavage, it is likely that Usp25m is the protease at the end of the signaling cascade in both pathways.

Finally, in order to better understand the mechanics of insulin resistance in an in-vivo model, we also hypothesize that using a model known to simulate the physiologic effects of insulin resistance, a high fat diet, cleavage of TUG will be impaired after treatment with insulin. If, as discussed in the introduction, TUG has an important role in tethering GSVs intracellularly in the basal state, and GSVs are then liberated upon insulin stimulation, there should be a difference in the abundance full length and proteolytic products of TUG after insulin stimulation. To look into this, we will examine TUG proteolysis in a mouse model that replicates insulin resistance. Together, this work aims to connect highly detailed mechanistic data gleaned from cultured cells with a well-established animal model of type 2 diabetes mellitus.

3. Methods

3.1 Reagents and cell culture

Polyclonal rabbit antisera directed against the TUG C-terminus have been previously described (95). Antibodies to GLUT4 were also previously described (95).

Other antibodies were purchased, including antibodies directed against the following: β-actin (ThermoFisher Scientific, catalog no. MA5-15739), Usp25 (Abcam ab187156), α-tubulin (Sigma T5168), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling catalog no. 97166), and Phospho-Acetyl-CoA Carboxylase (Cell Signaling, no. 3661). A number of plasmids were kind gifts. The plasmids to express myc-Usp25a and myc-Usp25m are from Dr. Gemma Marfany (96). Chemicals were obtained from MilliporeSigma unless otherwise noted.

3T3-L1 adipocytes, HEK 293, and HeLa cells were cultured in high-glucose DMEM GlutaMAX medium (Invitrogen) containing 10% bovine growth serum (BGS; EquaFetal) and plasmocin (Invitrogen). 3T3-L1 adipocytes that were infected with a puromycin resistance gene were also treated with puromycin (Sigma no. P9620) at 10 μg/mL. 3T3-L1 adipocytes were differentiated in 10% BGS with supplements, as described previously in the literature (95).

AMPK was activated in cell culture with either A-769662 (Cayman Chemical, no. 11900) at 100 μM or phenformin (Sigma no. P7045) at 4 mM. To increase calcium concentration, ionomycin (Cayman Chemical no. 11932) was used at 1 μM. For in vivo mice experiments, AICAR (Sigma, no. A9978) was used at a concentration of 0.25 mg/g to activate AMPK (75). All live mice work was conducted by Dr. Estifanos Habtemichael and Don Li.

For transient transfections of HeLa cells, mouse embryonic fibroblasts and 3T3-L1 adipocytes, the following protocol was used: for each 10 cm plate, Lipofectamine reagent (10 μL unless otherwise noted) was incubated with 0.25 mL DMEM for 5 minutes. The plasmid (4g, unless otherwise noted) to be transfected was also incubated

with 0.25 mL DMEM for 5 minutes. These two mixtures were then combined using mild mixing motion and incubated for 20 minutes. After incubation, the mixture was added drop wise to a 10 cm dish, and gently mixed. Unless otherwise noted, cells were harvested 48 hours after transfection.

For AMPK experiments, cells were serum starved in DMEM for 1 hour unless otherwise noted. Cells were lysed in TNET buffer as described by Belman et al. (95), the only difference being that 1% Nonidet P-40 was used in place of 1% Triton-100. SDS-PAGE and immunoblots were performed as described previously (95). Images were acquired on HyBlot film (Denville) using enhanced chemiluminescence (ECL) (Pierce). Densitometry of films was done using transillumination (Epson Perfection V700 scanner), and was quantified using ImageJ.

Coimmunoprecipitation was performed using the same buffer as described above. After lysis, anti c-Myc affinity gel was added, and incubated overnight at 4°C. After 6 washes in the same buffer, elution was done in glycine.

3.2. Mice

Animal protocols were approved by the Yale University Institutional Animal Care and Use Committee. All animals were handled and sacrificed by Dr. Estifanos Habtemichael and Don Li. The animals were housed at the Yale Animal Research Center at 22ºC (+/- 2 degrees), with light from 0700-1900 and dark from 1900-0700. Animals were given free access to chow (Harlan-Teklad 2018, 5% calories from fat, unless otherwise noted) and water. High fat diet mice were fed ResearchDiets D12492 (60% kcal from fat) for three weeks before sacrificing. 30 minutes after treatment with AICAR

or phosphate buffered saline (placebo), animals were sacrificed, and muscles were collected and flash-frozen in liquid nitrogen, and immediately stored at -80ºC. For Western blots, tissue was thawed, then lysed in 1% Nonidet P-40 lysis buffer as described above. Tissues were lysed for 3 minutes at 30 Hz (Qiagen TissueLyser II) in pre-chilled 4ºC clamps. Lysates were centrifuged at 13200 rpm for 15 minutes, and western blotting was conducted (62).

4. Results

4.1. Usp25m interacts with TUG

Previous results showed that insulin stimulates the cleavage of the 60 kDa TUG protein into an 18 kDa N-terminal product and a 42 kDa C-terminal product (53). Our data implicated Usp25 as a candidate protease that cleaves the intact TUG protein. Our experiments also showed that Usp25m (a muscle specific isoform of Usp25) expression increases in 3T3-L1 adipocytes as they differentiate from fibroblasts. These results were published in 2018 as part of a larger study (62).

My specific role in the project was to confirm that Usp25 interacts with TUG. The results of the coimmunoprecipitation experiments (Fig. 2) demonstrated that Usp25m did indeed interact with TUG, and that this interaction was specific to Usp25m, not the ubiquitous form of the protease, Usp25a (62, 97). The results of these experiments (Fig. 2) were published as Figure 1D in the above-referenced paper (62). Both the whole cell lysate and the fraction that was immunoprecipitated with anti-Myc antibody and eluted were immunobloted to show that absence of protein in the eluted fraction was not due to lack of protein in the whole cell lysate.

4.2. TUG cleavage differences in HFD and regular chow (RC) fed mice

Previous studies have shown that mice on a HFD have a whole-body insulin resistance phenotype (98). To investigate whether mice fed a HFD had impaired cleavage of TUG, wild-type mice were fed regular chow (RC) or a high fat diet (HFD) for three weeks. They were fasted, then treated with intraperitoneal injection of insulin-glucose solution or saline control. After 30 minutes, mice were euthanized, hindlimb muscles were isolated, and lysates were prepared and immunoblotted using an antibody to the TUG C-terminus. Immunoblots (Fig. 3) were made from the lysates of quadriceps. Samples from the mice fed a HFD had over a 2-fold decrease in cleavage of TUG compared with mice fed RC (Fig. 3). This result draws an important connection between in vitro studies describing the mechanism of GLUT4 retention and translocation and in vivo studies in a validated mammal model of insulin resistance.

4.3. Activated AMPK

Mice were treated with intraperitoneal AICAR, a known activator of AMPK. This was used at a concentration of 0.25 mg/g, dissolved in PBS, and injected intraperitoneally. PBS alone was used as a control. Mice were sacrificed 30 minutes after injections (99). Immunoblots were made from lysates of cardiac and skeletal muscle. There was a reduced abundance of intact TUG in lysates of the quadriceps muscle from mice with AMPK treated with AICAR, compared to controls treated with saline (not shown). With only 3 animals in each group however, this result was not statistically significant.

In cardiac muscle, animals treated with AICAR had significantly reduced intact TUG (Fig. 4). The short 30 minute period between the administration of AICAR and sacrificing the animals suggests that the observed reduction in TUG is an acute effect, possibly due to proteolytic processing. The 30 minute period would be too short to see an effect on gene expression. C-terminal TUG cleavage product was not visualized in muscle lysates, but was in cell culture. The reason for this difference could be either that the 42 kDa product was incorporated into the pellet with insoluble material from the preparation, or because of degradation.

We used two different methods to activate AMPK in cell culture, phenformin and a combination of A-769662 and ionomycin. In non-insulin responsive cells, specifically HeLa cells and mouse embryonic fibroblasts, we saw no difference in TUG cleavage between culture cells treated with these two methods of activating AMPK and non-treated controls, despite confirming that AMPK was active by measuring Phospho-Acetyl-CoA Carboxylase (pACC; not shown). We then repeated the experiment, this time using insulin responsive cells (3T3-L1 adipocytes), and still treating with A-769662 at 100 μM for one hour and ionomycin at 1μM for 20 minutes to increase the calcium concentration. Again, pACC was quantified to assess AMPK activation. A significant ~2-fold increase in cleavage of TUG was seen in cultured 3T3-L1 adipocytes (Fig. 5). A cleavage product of 42 kDa detected by an antibody raised against the C-terminal portion of the TUG protein was observed, which is the same fragment detected in insulin-stimulated TUG cleavage, implying that end of the signal cascade in both AMPK and insulin mediated TUG cleavage is the same. AICAR is commonly used experimentally to activate AMPK, but its complicated pharmacology as well as off target effects can make

determining the mechanisms difficult (69). One rationale of using a more direct AMPK activator, A-769662, for cell culture studies is that the results can more reliably give information about the mechanism of GLUT4 translocation.

Notably, previous results had shown that ischemia activates AMPK in cardiac muscle, and that AMPK-deficient mouse hearts expressing a kinase dead $\alpha 2$ catalytic subunit had reduced uptake of glucose into the cardiac muscle (100). More recent results had also showed that TUG might mediate this effect on glucose uptake (89).

In primary tissue, processing of the lysate frequently causes the 42 kDa C-terminal cleavage product to be undetectable, likely because this product is with the nuclear fraction after centrifugation. Thus, in primary tissue, the change in abundance of the full-length TUG protein is used to assess cleavage, as in Fig. 4. In contrast, in cultured 3T3-L1 adipocytes, the baseline abundance of full-length TUG is so great that the most effective method for assessing proteolysis of TUG is by measuring the abundance of cleavage products, usually the 42 kDa C-terminal product.

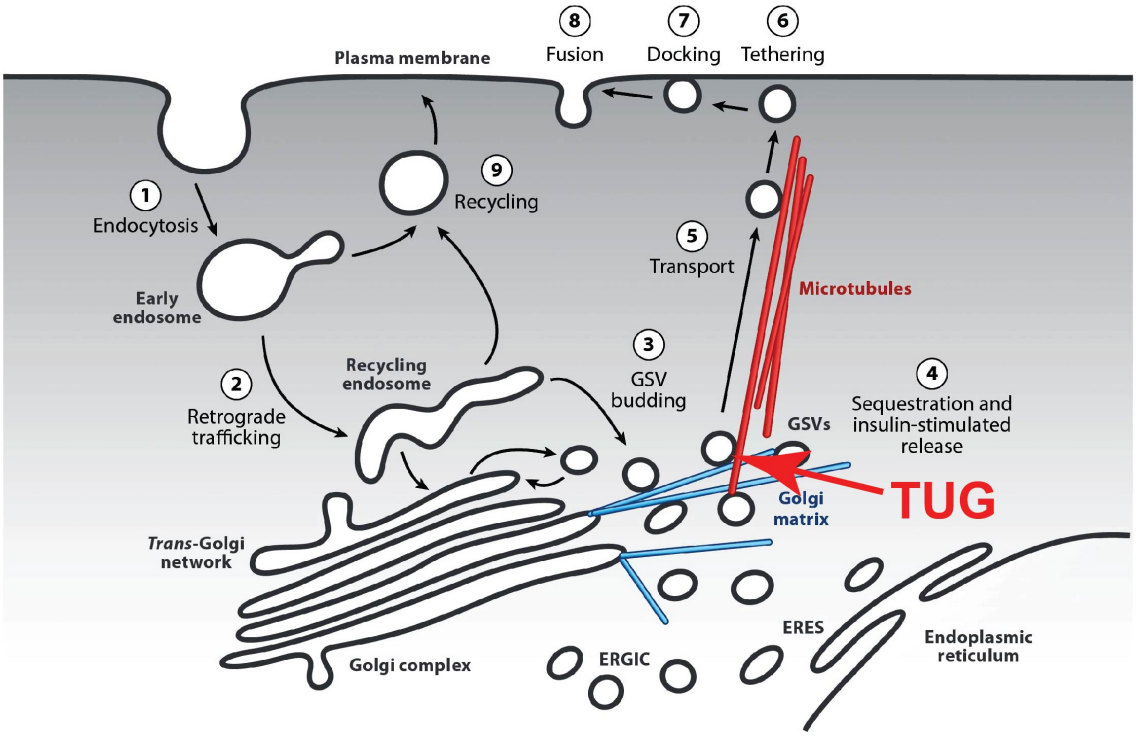

Figure 1: Model of GLUT4 trafficking. After endocytosis (1), GLUT4 enters the early endosome and proceeds to the recycling endosome and trans-Golgi network by a retrograde trafficking pathway (2). GLUT4 storage vesicles (GSVs) bud from the recycling endosome and/or from Golgi membranes (3) and are sequestered by the action of TUG, which links the vesicles to the Golgi matrix (4). Insulin stimulates TUG cleavage to release the GSVs, which are transported along microtubules (5) to the plasma membrane. At the plasma membrane, the vesicles are tethered (6), dock (7), and fuse (8) in processes that are also regulated by insulin. GLUT4 that is endocytosed in the presence of ongoing insulin exposure recycles back to the plasma membrane directly from endosomes (9) and bypasses the GSV sequestration mechanism. Diagram adapted from Ref. (36).

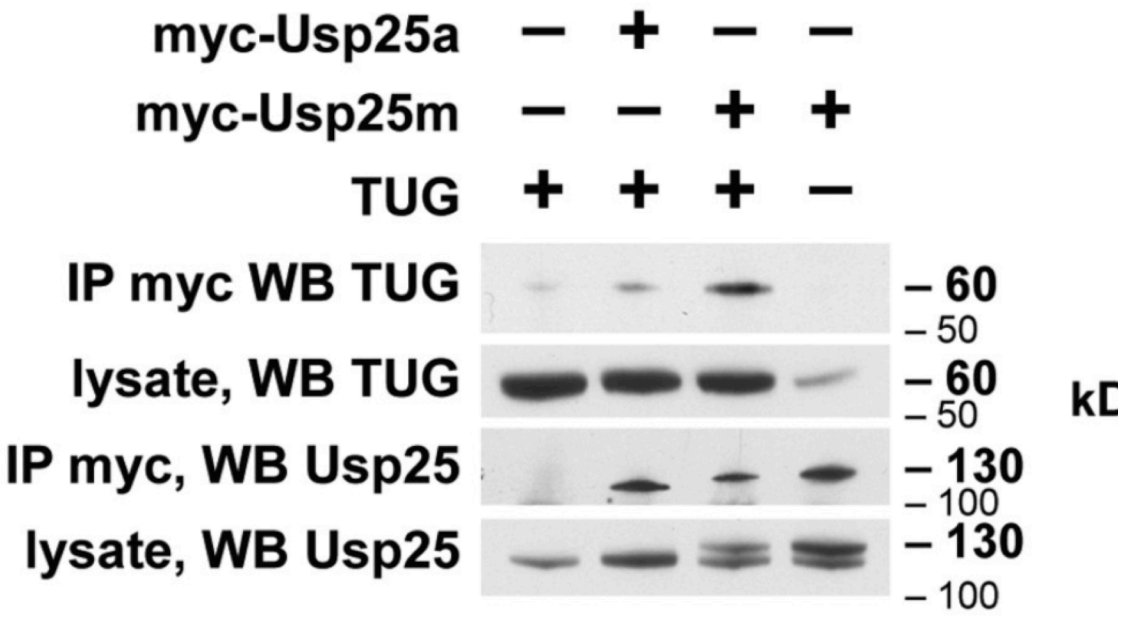

Figure 2: TUG interacts preferentially with the muscle splice form of Usp25, Usp25m, compared to the more widespread variant, Usp25a. The indicated proteins were transfected in 293 cells. Cells were lysed and the myc-tagged Usp25 proteins were immunoprecipitated. Eluates were immunoblotted to detect TUG. Published as Figure 1D of Habtemichael et al. 2018 (62).

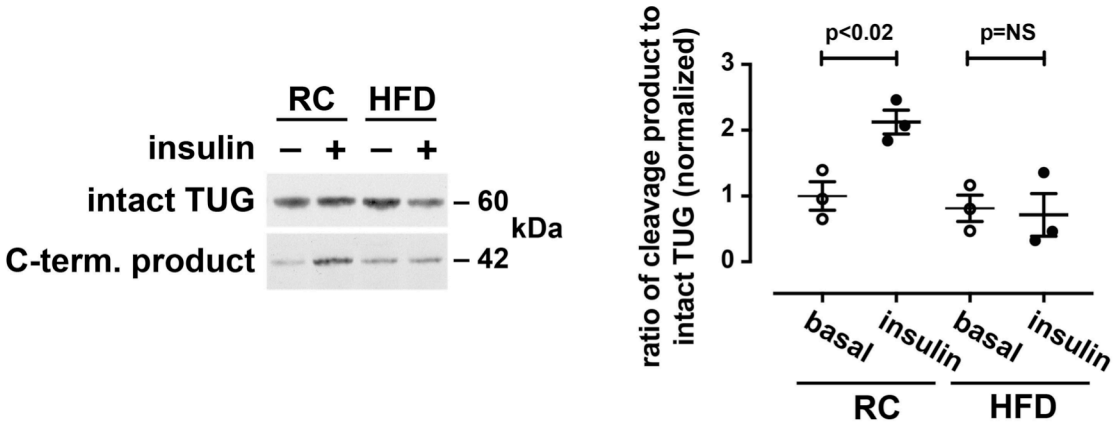

Figure 3: Insulin-stimulated TUG endoproteolytic cleavage is reduced in muscles of mice fed a high-fat diet, compared to those maintained on regular chow. Mice were fed regular chow (RC) or a high fat diet (HFD) for three weeks. They were fasted, then treated with intraperitoneal injection of insulin-glucose solution, or saline control. After 30 min., mice were euthanized, hindlimb muscles were isolated, and lysates were prepared and immunoblotted using an antibody to the TUG C-terminus. Representative immunoblots are shown at left. Data from replicate experiments were quantified and are plotted on the right.

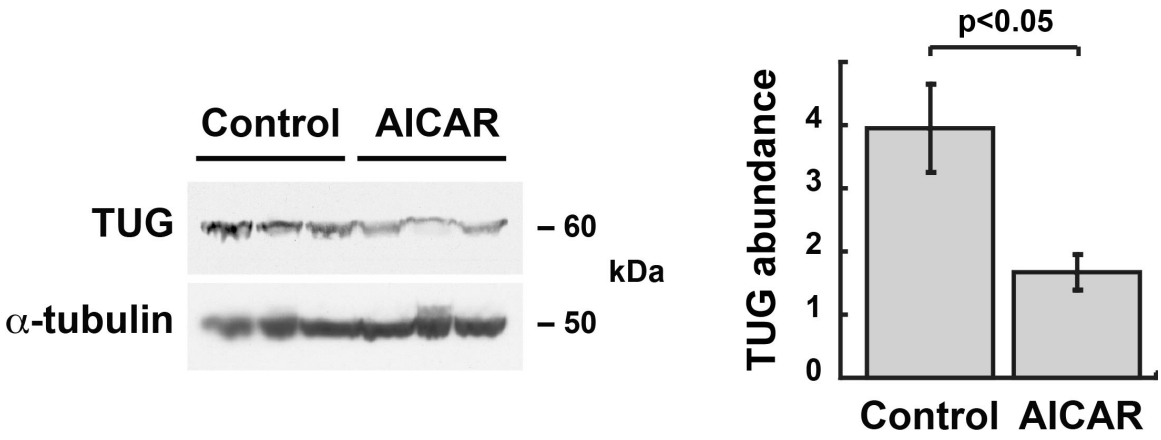

Figure 4: Mice treated with intraperitoneal AICAR have decreased abundance of intact TUG in cardiac muscle, consistent with AMPK-stimulated TUG cleavage. Mice were fasted, then treated by intraperitoneal injection of AICAR or saline control, as described in the Methods section. After 30 minutes, mice were sacrificed, lysates were prepared from hearts, and immunoblots were done as indicated (left panel). Although TUG cleavage products were not observed, the abundance of intact TUG was reduced after AICAR treatment. Densitometry was done to quantify the effect, and is shown in the right panel.

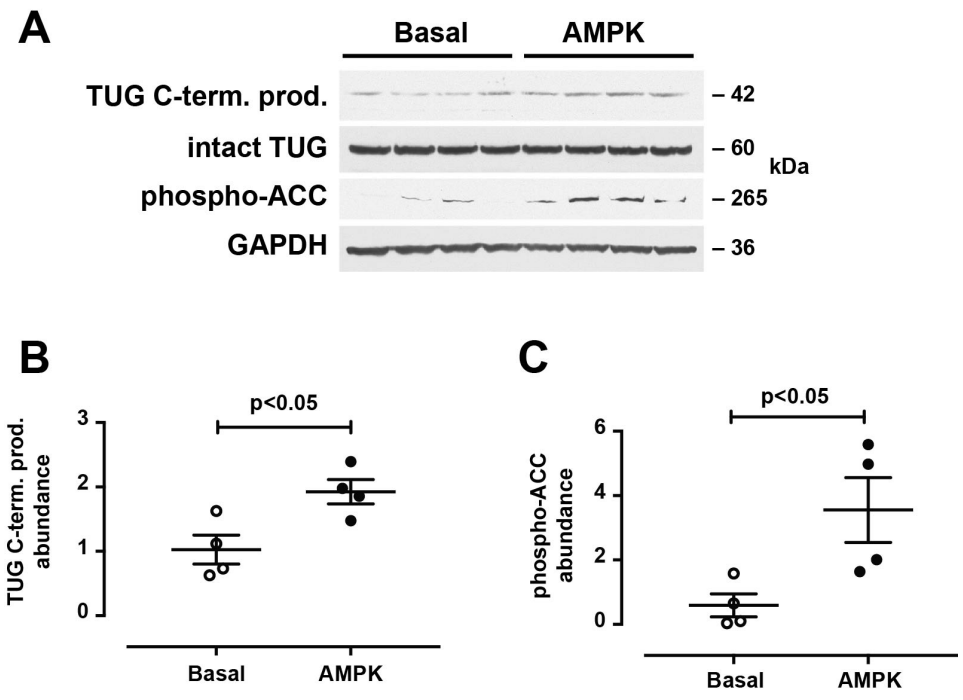

Figure 5: A-769662 and ionomycin treatment causes activation of AMPK and TUG cleavage in adipocytes. 3T3-L1 adipocytes were cultured in serum free media and left untreated (Basal) or stimulated using A-769662 and ionomycin. Cells were then lysed and analyzed by SDS-PAGE and immunoblotting to detect the TUG C-terminal cleavage product, intact TUG, phosphorylated Acetyl-CoA carboxylase (ACC), and a loading control, GAPDH. A. Representative immunoblots are shown. B. The abundance of the TUG C-terminal product was quantified using densitometry, and is plotted. C. The abundance of phosphor-ACC was quantified and is plotted, and confirms that AMPK was activated in the treated cells.

5. Discussion

5.1. Usp25m interacts with TUG

The results presented here help to characterize the mechanisms downstream of insulin signaling and AMPK activation that trigger GLUT4 translocation to the plasma membrane. Coimmunoprecipitation experiments show that TUG can be purified with the Usp25m isoform, but not the Usp25a isoform. This work contributes to the characterization of the proteolysis that results in cleavage of TUG.

Further work done by other members of Jonathan Bogan's laboratory strongly supports a model in which Usp25m is the protease responsible for insulin-regulated cleavage of the TUG protein (62). To put the data presented in Fig. 2 into a broader context, other results from Jonathan Bogan's laboratory have shown that the expression of Usp25m dramatically increases as 3T3-L1 adipocytes differentiate from fibroblasts, and that by day 4 of differentiation, Usp25m was near a maximal level. Furthermore, Usp25m could be copurified with TUG in 3T3-L1 adipocytes, and treatment with insulin caused the disassociation of Usp25m from TUG (62). Subcellular fractionation experiments also demonstrated that Usp25m co-localizes with GSVs. Specifically, insulin-regulated aminopeptidase (IRAP), which is present in GSVs, was mobilized out of the light microsome (LM) into the plasma membrane (PM) fraction, and Usp25 was mobilized to the cytosolic fraction (62). Lastly, knockdown of Usp25m in 3T3-L1 adipocytes using short hairpin RNA (shRNA) significantly ablated insulin-stimulated cleavage of the 60 kDa TUG protein in the 42 kDa C-terminal fragment and the 18 (or larger if modified) kDa N-terminal fragment (62).

5.2. HFD inhibits TUG cleavage

As discussed in the introduction, overnutrition is a proximate cause of insulin resistance, but the mechanisms are multifactorial and not well understood. Previous data supported a model in which TUG is cleaved in response to insulin stimulation, and, in 3T3-L1 adipocytes, Usp25m is the protease that cleaves the TUG protein (53, 62). Using a validated model of insulin resistance (3 weeks of high fat diet), our data demonstrated that TUG cleavage after insulin stimulation is drastically impaired in mice that are insulin resistant. Insulin resistant mice had over a two-fold decrease in insulin stimulated TUG cleavage compared to normal chow fed mice. Significantly reduced cleavage of TUG in an animal model that reproduces the insulin resistance phenotype offers support for TUG as a key tether that regulates GLUT4 abundance in the plasma membrane of adipocytes and myocytes, and thus serum glucose and insulin levels. A notable limitation of this work is that the mechanism of impaired TUG cleavage was not determined; it remains unclear if changes in the amount or activity of Usp25m affect TUG cleavage. Taken together with previous work, these results support a model of insulin-stimulated TUG cleavage as a key regulatory step for GLUT4 translocation to the plasma membrane that is deranged in an insulin resistant state.

5.3. AMPK

Our results suggest that in the two major insulin-responsive cell types important for post-prandial glucose disposal, AMPK mediates cleavage of the TUG protein. AMPK has been mostly studied in skeletal muscle and in response to exercise. Thus, the results presented here in adipocytes contribute to the broader understanding of the similarities

between insulin and non-insulin dependent mechanisms of GLUT4 translocation. Both in vivo (cardiac myocytes; Fig. 4) and in cultured adipocytes (Fig. 5), activation of AMPK stimulated TUG cleavage. The latter result confirms previous work that showed ischemia caused cleavage of TUG in cardiac myocytes (89). The observation that activated AMPK causes cleavage of TUG in cultured 3T3-L1 adipocytes is significant because adipocytes are a well-established model cell type in diabetes research, amenable to modification for further study, and more established and reliable than myocyte cell lines.

Going forward, the signal cascade that controls AMPK-stimulated TUG cleavage remains the focus of active research. AMPK is a ubiquitous kinase and TUG proteins are expressed in numerous cell types, but activated AMPK does not cause cleavage of TUG in mouse embryonic fibroblasts or HeLa cells (see results in section 4.3). Usp25m is known to be the protease that cleaves TUG in adipocytes downstream of insulin (62). According to the results presented in this thesis, Usp25m also likely mediates TUG cleavage downstream of AMPK in adipocytes and myocytes. With both AMPK-mediated and insulin-mediated TUG cleavage, the same size proteolytic products are formed, supporting the hypothesis that Usp25m is an important protein not present at sufficient levels in non-insulin responsive cells.

Another area of active research is aimed at determining whether activated AMPK directly phosphorylates TUG, or if the interaction is indirect. If the interaction is direct, at what residue is TUG phosphorylated? As noted above, preliminary data suggest that T57 is a likely candidate, based on in vitro phosphorylation experiments using recombinant proteins. One way to study this would be to knock out TUG in 3T3-L1 adipocytes, and infect these cells with mutated forms of TUG – specifically, mutating the threonine

residue to alanine (T57A), which would block phosphorylation, and in another sample to glutamic acid (T57E), which may mimic phosphorylation. While knock out adipocytes are being generated, overexpression of the T57A mutant would be a good next step to study this effect. 3T3-L1 adipocytes contain endogenous TUG, however if the T57A mutation disrupts AMPK-stimulated proteolysis, then stabilization of this protein should still be observed. In this way, it may be possible to determine if AMPK and insulin both activate cleavage at the same site, as the results presented above may suggest.

Lastly, the physiologic role of increased glucose transport in the heart during states of ischemia could be explained by an increased demand for glucose to produce ATP. When oxygen, and consequently oxidative phosphorylation, are limited, anaerobic glycolysis is a low efficiency path to produce ATP. However, another explanation is also possible. As detailed in the introduction, GLUT4 is one cargo on GSVs, along with IRAP and LRP1. Perhaps GLUT4 is simply an extra cargo protein that is released to the cell surface along with the primary cargo. In ischemia, the problem is lack of blood flow, which not only decreases ATP production due to lack of glucose and fatty acids, but also decreases disposal of metabolic waste and perhaps, most importantly, stops the highest yield ATP formation step, oxidative phosphorylation. Given both the key role of oxygen, and the fact that in GSVs, there are about twice as many molecules of IRAP as GLUT4, it seems more likely that degradation of vasopressin and subsequent vasodilation would be the most important physiologic responses in the ischemic heart (101). These effects are likely not mutually exclusive; increased GLUT4 expression and thus glucose in cardiac muscles may well be useful during ischemia.

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