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GIV as a Regulator of GLUT4 Trafficking:

A Novel Target for Modulating Insulin Resistance in Diabetes

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

Shabnam Pedram

Committee in charge:

Professor Pradipta Ghosh, Chair
Professor Amy Kiger, Co-Chair
Professor Aaron Coleman
Professor Marilyn Farquhar

2013

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Co-Chair

Chair

University of California, San Diego

2013

I dedicate this thesis to my family, for their support and encouragement throughout this process.

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ABSTRACT OF THE THESIS

GIV as a Regulator of GLUT4 Trafficking:

A Novel Target for Modulating Insulin Resistance in Diabetes

by

Shabnam Pedram

Master of Science in Biology

University of California, San Diego, 2013

Professor Pradipta Ghosh, Chair

Professor Amy Kiger, Co-Chair

Insulin resistance (IR) and Diabetes are well known risk factors for many cardiovascular diseases. IR is a result of dysregulated signaling downstream of the insulin receptor (InsR). Binding of insulin to its receptor triggers the activation of IRS1, which in turn activates the PI3K-Akt pathway and trimeric G protein, Gi, both prerequisites for the translocation of glucose transporters, GLUT4, to the plasma membrane (PM) and glucose uptake. In IR, circulating fatty acids activate PKCθ, which phosphorylates and

antagonizes IRS1-mediated responses. Efforts at reinstating sensitivity in IR simply by targeting the known players within the insulin signaling pathway have failed, and thus, the search for new targets continues. GIV (a.k.a. Girdin) has recently been identified as a multimodular signal transducer and a guanine nucleotide exchange factor (GEF) for Gαi, which enhances the PI3K-Akt pathway downstream of the InsR. Here we show that activation of Gi and PI3K-Akt signals via GIV's GEF function is critical for enhancing IRS1 activation and GLUT4 translocation to the PM. Phosphoinhibition of GIV's GEF function by PKCθ antagonized GIV-dependent IRS1 and Akt activation and translocation of GLUT4 to the PM. We conclude that GIV is one of the key mediators of insulin response that activates PI3K-Akt and Gi to maintain insulin sensitivity, and that its phosphoinhibition by the fatty-acid-PKCθ pathway contributes significantly to the development of IR. Future work unraveling how GIV-GEF mediates insulin response, and how its inhibition can trigger IR will offer a new target to combat IR and reverse/halt the progression of cardiovascular diseases.

I:

Introduction

1. Insulin Resistance

1.1 Background and Significance

Under normal conditions, the pancreas releases insulin into the bloodstream following a rise in blood glucose levels. Insulin signals cells in muscle, liver, and adipose tissues to take up glucose from the extracellular environment so as to bring blood glucose back down to homeostatic levels. In insulin resistant patients, however, cells become less responsive to insulin and show a reduced ability to transport glucose into tissues. In insulin resistance (IR), pancreatic beta cells secrete excess amounts of insulin in order for cells to detect and respond to insulin. Eventually, beta cells fail to keep up with the body's continuous demand for insulin and there is a rise in blood glucose (hyperglycemia), setting the stage for the development of Type 2 Diabetes Mellitus (T2DM) and cardiovascular disease (CVD) (Figure 1). IR itself, even in the absence of other disease, is an independent risk factor for stroke, congestive heart failure, and inflammation-driven progression of atherosclerosis, which further increases the risk for development of coronary artery disease and strokes. The major causes of IR are obesity, physical inactivity, genetics, aging, and sleep problems such as apnea. IR is also recognized as a key pathophysiologic component in patients with a family history of diabetes, personal history of gestational diabetes, suffering from polycystic ovarian syndrome (PCOS), and obese patients (particularly abdominal obesity) (NIH 2012).

Pathogenesis of IR stems from defects in key players within the insulin signaling cascade that trigger aberrations in the entire signaling network downstream of insulin receptors (InsR and IGF1R). Therefore, due to the far-reaching and detrimental effects of IR in health, studying the insulin signaling pathway involved in insulin response can

shine light on key molecular targets for drug development. Already there are insulin sensitizer drugs, Thiazolidinediones (TZD's), which work to lower blood sugar by increasing cell sensitivity to insulin in muscle, fat, and liver tissues. However, TZD's are not successful in every patient and they are associated with many health risks or side effects, such as increased risk of heart disease and congestive heart failure, weight gain, fluid retention (Nesto et al. 2004), and increased risk of bone fractures (Riche and King 2010). Therefore, it is critical to continue searching for key players within the insulin response pathway that can be either targeted for treatment of IR, or can serve as predictors of response to therapy with TZDs.

The work presented in this thesis forms the foundation and represents a part of a much larger scope of study which is focused on elucidating the role of a novel multidomain signal transducer called GIV/Girdin in insulin response. Preliminary research (outlined in the sections below) conducted in the lab points to GIV as an enhancer of insulin sensitivity via its interactions with critical signal cascade components downstream of the Insulin Receptor (InsR). This research strives to elucidate the mechanism by which GIV can enhance insulin sensitivity and whether phosphomodification and dysregulation of GIV could result in IR.

1.2 Activation of the InsR→IRS1→PI3K→Akt axis of signaling is required for rapid translocation of GLUT4 to the plasma membrane.

The mechanism of insulin response (Figure 2) begins with binding of the hormone insulin to the insulin receptor (InsR) and concurrent receptor dimerization. The InsR is a tyrosine kinase receptor (RTK) that when bound to its ligand, will become activated and autophosphorylated on tyrosine residues at its cytoplasmic tails. Phosphorylated insulin

receptor becomes a docking site for downstream effectors, and specifically in the insulin signaling pathway, activated InsR will phosphorylate critical tyrosine residues on the Insulin Receptor Substrate-1 (IRS-1) molecule. Activated IRS-1 will recruit and activate PI3K, a phosphoinositide 3-kinase, which converts phosphatidylinositol 4,5-bisphosphate (PIP2), a membrane lipid, to phosphatidylinositol- (3,4,5)-triphosphate (PIP3). PIP3 will recruit and activate Akt (serine/threonine protein kinase B), and activated Akt promotes expression and translocation of glucose transporter channels to the plasma membrane (PM) and subsequent uptake of glucose into the cell (Saltiel and Kahn 2001).

1.3 Glucose uptake requires translocation of GLUT4 channels to the PM

GLUT4 channels are glucose transporters found primarily in muscle and fat tissues, and upon insulin stimulation, these twelve-transmembrane channels, which are packaged in GLUT4 storage vesicles (GSVs), are translocated to the plasma membrane (PM) where they take up glucose from the bloodstream by facilitated transport. Insulin-stimulated activation of Akt directs translocation of GLUT4 channels to the PM (Saltiel and Kahn 2001). It is well established that the PI3K pathway promotes GLUT4 translocation to the PM (Martin et al. 1996), and inactivation of this same pathway blocks GLUT4 translocation to the PM (Cheatham et al. 1994), therefore establishing the PI3K pathway as a regulator of GLUT4 translocation and hence glucose uptake.

1.4 Insulin sensitivity requires balanced signaling between the PI3K-Akt and the MAPK-ERK signaling pathways; tilting this balance heralds insulin resistance

The InsR regulates two distinct cellular signaling pathways, the PI3K-Akt pathway and the MAPK-ERK pathway (Yu et al. 2002; Thirone, Huang, and Klip 2006). Research findings have demonstrated that insulin stimulation under normal conditions

results in a parallel and dichotomous activation of the PI3K-Akt and MAPK-ERK pathways, while in IR there is a differential response resulting in downregulation of PI3K signaling and no change, or possibly even an upregulation due to hyperinsulinemia of MAPK-ERK signaling (Cusi et al. 2000; Thirone, Huang, and Klip 2006; Yu, Gao, and Ma 2011), seen in Figure 3.

Although the exact molecular mechanism behind this PI3K-Akt/ MAPK-ERK dichotomy of signaling cascades has not yet been identified, it is well accepted that IRS-1 is critical to insulin response via activation of the PI3K pathway. According to Thirone *et al.,* IRS-1 expression correlates with insulin-induced activation of the PI3K-Akt pathway, and the up-regulation of GLUT4 translocation, and subsequent uptake of glucose into cells. Furthermore, myotubes of IRS-1 knockout mice develop IR, due to a loss in PI3K-Akt activity and down-regulation of insulin-induced GLUT4 translocation and glucose uptake (Thirone, Huang, and Klip 2006). However, no link has been identified to date connecting IRS-1 activation to the mechanism of GLUT4 translocation.

1.5 Activation of trimeric Gi is required for physiologic insulin response, PI3K-Akt activation, GLUT4 translocation and glucose uptake

In insulin response, InsR has also been shown to work in conjunction with heterotrimeric Gαi protein during insulin-induced GLUT4 translocation and glucose uptake. When Gαi is inactivated by pertussis toxin, via ADP-ribosylation, there is a decrease in insulin sensitivity (downregulated PI3K-Akt) and a decrease in GLUT4 translocation (Ciaraldi and Maisel 1989). At the same time, increased concentration of insulin to the cell decreases ADP-ribosylation of Gi proteins, therefore making them more accessible for signal transduction (Rothenberg and Kahn 1988). Studies have also

shown that InsR promotes phosphorylation of Gi at the alpha subunit (Krupinski et al. 1988; O'Brien et al. 1987), thereby strengthening the connection between InsR and Gi proteins. However, despite numerous clues that insulin can activate Gi, the fundamental question as to how that occurs in cells remains unknown, and the molecular linker that connects InsR and Gαi, and how such complexes enable GLUT4 translocation, is not fully understood.

1.6 Protein Kinase C- Theta (PKCθ) antagonizes the InsR→IRS1→ PI3K→Akt axis of signaling and triggers insulin resistance in skeletal muscles

Yet another regulator of insulin signaling is Protein Kinase C- Theta (PKCθ), a novel isoform from the class of PKCs. Multiple groups (Gao et al. 2007; Yu et al. 2002; Kewalramani et al. 2011; Haasch et al. 2006) have unequivocally documented the existence of a direct link between PKCθ activation and the development of IR (Haasch et al. 2006). In a study done by Yu *et al.*, rats were infused with a lipid emulsion that increased cellular acetyl Co-A and diacylglycerol (DAG) concentrations (Yu et al. 2002), which are known to activate PKCs and lead to the development of IR (Gao et al. 2007). Yu *et al*. observed that PKCθ phosphorylates IRS-1 at Ser307, which leads to decreased tyrosine phosphorylation on IRS-1 and decreased IRS-1-associated PI3K activity (Yu et al. 2002). Subsequently, Kewalramani *et al.* showed that PKCθ siRNA knockdown in insulin resistant myoblasts (precursor muscle cells) reversed IR by increasing phosphorylated Akt and GLUT4 translocation to the cell surface (Kewalramani et al. 2011). Although these findings identified PKCθ as a crucial player in the genesis of IR, other possible substrates through which PKCθ may exert such a strong central role remains unknown.

2. GIV (a.k.a, Gα Interacting, Vesicle-associated protein; Girdin) GIV is a multidoman PI3K-Akt signal transducer and a non-receptor GEF for Gαi.

2.1 GIV serves as a nonreceptor GEF (Guanine Nucleotide Exchange factor) for Gαi proteins

GIV is a large, 220 kilodalton (kDa), multidomain protein that was first discovered based on its ability to bind Gαi3 (Le-Niculescu et al. 2005). G proteins are composed of 3 subunits, Gα, Gß, and Gγ, and they are guanine nucleotide binding proteins that serve as key molecular signal transducers, transmitting signals from activated receptors at the surface of a cell to downstream effectors. When bound to GTP, G proteins are active, and when bound to GDP, G proteins are inactive. Therefore, they serve as molecular switches in signal cascades and their activity is regulated by molecules that can hydrolyze GTP to GDP (GTPases) and those that can exchange a GDP for a GTP (GEF proteins) (Oldham and Hamm 2008). GIV, in fact, serves as a non-receptor GEF for Gαi, and this GEF domain was discovered based on similarity to an evolutionary conserved motif in GIV that shares the same sequence as a synthetic GEF peptide KB-752 (Garcia-Marcos, Ghosh, and Farquhar 2009). The GEF motif, which lies at the C-terminus of the GIV protein, allows GIV to bind to and activate Gαi, resulting in downstream signaling events.

2.2 Activation of Gαi by GIV's GEF motif serves a variety of functions, all through activation of Class I PI3Ks.

GIV has a diverse set of roles-- it enhances Akt signals (Anai et al. 2005) and couples this to actin remodeling at the leading edge of migrating cells (Enomoto et al.

2005; Enomoto, Ping, and Takahashi 2006), enhances macrophage chemotaxis (Ghosh et al. 2008), modulates mitosis (Ghosh et al. 2010), autophagy (Garcia-Marcos et al. 2011), and tumor progression and invasion (Ghosh, Garcia-Marcos, and Farquhar 2011). GIV's role in such a diverse set of biological processes and in varying cell types is consistent with the fact that GIV can modulate signals initiated by an equally diverse group of receptors and ligands. It has been demonstrated that two classes of receptors utilize GIV for enhancement of Akt and for triggering cell migration; receptor tyrosine kinases (RTKs; e.g., EGFR and InsR) and G-protein coupled receptors (GPCRs; e.g., LPAR) (Enomoto et al. 2005; Enomoto, Ping, and Takahashi 2006; Garcia-Marcos et al. 2010; Garcia-Marcos, Ghosh, and Farquhar 2009; Ghosh et al. 2008). Thus, GIV serves as a common platform that links multiple ligand-activated receptors to actin, Akt, and Gαi3.

Mechanistically, GIV enhances the PI3K-Akt signaling axis by two mechanisms: a) GIV activates Gαi3 via its GEF motif and simultaneously releases the Gβ/γ subunits, which bind and activate PI3K (Enomoto, Ping, and Takahashi 2006; Garcia-Marcos et al. 2010); b) GIV is phosphorylated at two tyrosine residues (Tyr-1764/1798) in the C-terminus by RTKs and non-RTKs which triggers activation of PI3K and promotes tumor cell migration (Lin et al. 2011). These mechanisms result in synergistic activation of the PI3K-Akt pathway via GIV.

2.3 PKC-theta (PKCθ) phosphorylates GIV and inactivates GIV's GEF function

It has been demonstrated that GIV's ability to activate Gαi3 can be effectively and selectively abolished by mutating critical residues within the GEF motif. One such mutation was identified to be Phenylalanine 1685 to Alanine (F1685A), which resulted in

consequent downregulation of Akt activity and cell motility (Garcia-Marcos, Ghosh, and Farquhar 2009; Garcia-Marcos et al. 2012). However, nothing was known about how GIV's GEF function is regulated in physiology. In a recently completed project which was ongoing at the time I entered the lab, and on which I assisted (Lopez-Sanchez et al. 2013), it was reported that PKCθ (theta), a novel Protein Kinase C, downregulates GIV's GEF function by phosphorylating Ser(S)1689 located within GIV's GEF motif. It was demonstrated that PKCθ specifically binds and phosphorylates GIV at S1689, and this phosphoevent abolishes GIV´s ability to bind and activate Gαi. HeLa cells stably expressing the phosphomimetic mutant of GIV, GIV-S1689→D (SD), are phenotypically identical to those expressing the GEF-deficient F1685A (FA) mutant: Actin stress fibers are decreased, cell migration is inhibited whereas cell proliferation is triggered, and Akt activation is impaired downstream of both the lysophosphatidic acid (LPA) receptor, a GPCR, and the insulin receptor. These findings demonstrated that phosphorylation of GIV by PKCθ provides a novel negative feedback loop for regulating the GEF function of GIV and terminating its functions.

2.4 G proteins regulate activation of PKCθ

In the context of PKCθ signaling, it is noteworthy that the heterotrimeric Gi family of GTP-binding proteins can indirectly activate the kinase (Wu, Katz, and Simon 1993). Studies have shown that Gß/γ heterodimers activate phospholipase C (PLC), an enzyme that cleaves inositol phospholipids into DAG, an activator of the novel PKCθ isoform (Nishizuka 1995). As mentioned earlier, active PKCθ was found to phosphorylate GIV at S1689 and inhibits the GEF function of GIV in physiology, and subsequently downregulates the PI3K-Akt pathway (Lopez-Sanchez et al. 2013). Based

on this information (sections 2.3 and 2.4) we hypothesize that GIV may add a key and complex regulatory layer to the physiologic insulin response and GLUT4 translocation by functioning at the intersection of InsR, G proteins, and PKCθ because GIV's GEF motif directly binds and activates G proteins, which may affect PKCθ activity, and which in turn can phosphorylate and inhibit GIV's GEF function.

2.5 GIV's GEF motif is required for recruitment of Gi to InsR and its subsequent activation

The importance of GIV-dependent Gi activation downstream of receptor tyrosine kinases (RTKs) and the consequences of such activation has been shown to play a critical role in multiple cellular functions in physiology and disease such as, actin remodeling, mitosis, migration, autophagy and cancer cell invasion/metastasis (Enomoto, Ping, and Takahashi 2006; Garcia-Marcos et al. 2011; Ghosh et al. 2010; Ghosh et al. 2008). However, the mechanism by which GIV links receptor activation to G protein activation remained unknown. In a work recently concluded in my mentor's lab Lin et al. (Lin et al. Submitted (2013)), reported the discovery of a unifying mechanism that allows GIV to serve as a common platform directly linking many ligand-activated RTKs to activation of G proteins and defined the structural and biochemical basis for activation of G proteins by RTKs like InsR. They demonstrated that GIV is a unique SH2 adaptor which binds autophosphorylated cytoplasmic tails of multiple RTKs, and extends its GEF activity to link trimeric G proteins to ligand-activated growth factor receptors. Upon recruitment to EGFR or InsR, GIV recruits and activate Gi proteins via its GEF domain. The second consequence of such direct interaction between GIV and RTKs is that RTKs like InsR can subsequently phosphorylate GIV at two critical tyrosines, which directly bind

p85α(PI3K) and activate the Class 1 PI3Ks (Lin et al. 2011). It was demonstrated (Lin et al. 2011; Lin et al. Submitted (2013)) that binding of GIV and its subsequent tyrosine phosphorylation by EGFR or InsR are required for efficient recruitment of p85α(PI3Ks) to these receptors, activation of PI3K, production of PIP3 at the PM, and subsequent recruitment and activation of Akt kinase.

The other consequence of GIV binding the autophosphorylated cytoplasmic tails of RTKs is that it can compete for binding sites with other SH2 adaptors, a family of signaling intermediates that help shape the signaling cascades downstream of ligand-activated RTKs. It was shown (Lin et al. Submitted (2013)) that GIV competes with SH2 adaptors Shc1 and SHP1 for the same phosphotyrosines on EGFR tail, and that recruitment of GIV to EGFR suppresses the recruitment of Shc1 and SHP1. Such a profile of adaptor recruitment directly affects the duration of receptor activation (i.e., autophosphorylation), receptor trafficking and degradation, and activation of the mitogenic MAPK-ERK cascade. Because GIV directly binds InsR much like it binds EGFR, we hypothesize that binding of GIV to InsR might similarly skew the binding of some of the major adaptors (like IRS1, PI3K, SHP1, or Shc1).

2.6 Presence or absence of GIV's GEF function is a key determinant of whether the PI3K-Akt or the MAPK-ERK pathways are enhanced downstream of the InsR.

Interestingly, research done by Garcia-Marcos *et al*. has demonstrated that GIV is involved in regulating the dichotomous balance between the PI3K-Akt/mTOR and MAPK-ERK pathways in cellular response to insulin (Garcia-Marcos et al. 2011). In research to identify GIV's role in regulating cellular autophagy, Garcia-Marcos *et al.*

discovered that upon insulin stimulation, over-expression of GIV enhances the PI3K-Akt/mTOR pathway (identified by expression of phosphorylated Akt, S6K, and S6 ribosomal protein), which promotes intercellular anti-autophagic signals, and downregulates the MAPK-ERK pathway (signified by levels of active ERK) and inhibits cellular autophagy. Conversely, dysfunctional GIV (a GEF mutant) results in the opposite cell signaling response: downregulated PI3K-Akt/mTOR pathway and upregulated MAPK-ERK pathway (Garcia-Marcos et al. 2011). As mentioned earlier, this dichotomy of cell signaling is also characteristic of insulin response in which Akt levels are upregulated and ERK levels are downregulated, and the signaling dichotomy is reversed in IR.

3. Hypothesis and aims

In summary, GIV's GEF motif is required for enhancement of the PI3K-Akt pathway (Garcia-Marcos et al. 2012), a key pathway which confers insulin sensitivity (Cheatham et al. 1994; Hernandez, Teruel, and Lorenzo 2001; Martin et al. 1996). PKCθ, which is implicated in the development of IR (Haasch et al. 2006), phosphorylates and inhibits GIV´s GEF activity and the PI3K-Akt pathway. In this current study, we have taken advantage of a widely accepted and well-studied tool for monitoring GLUT4 translocation to the PM by use of HA-GLUT4-GFP construct (Dawson et al. 2001; Zhao et al. 2009) to study GIV's role in regulating GLUT4 translocation and its role in the modulation of key molecular components of the insulin signaling pathway. We hypothesize that GIV's GEF motif is required for insulin sensitivity and that when PKCθ phosphorylates and inhibits the GEF activity (mimicked by GIV-SD mutant), GIV-

dependent PI3K-Akt signals are suppressed, GLUT4 fails to translocate to PM in response to insulin, and IR sets in.

II:

Results

1. HA-GLUT4-GFP: A fluorescence-tagged chimera for visualization and quantification of GLUT4 translocation

To study GLUT4 translocation upon ligand stimulation in HeLa cells, we used an HA-GLUT4-GFP plasmid construct, obtained from the Dawson Lab (Dawson et al. 2001), which is a chimeric protein of GLUT4 flanked by a hemagglutinin (HA) epitope tag on the N-terminal exofacial loop and a green fluorescent protein (GFP) on the C-terminus (Figure 4A). This construct has previously been used extensively in both 3T3-L1 cells (Zhao et al. 2009; Dawson et al. 2001) and in HeLa cells (Haga, Ishii, and Suzuki 2011; Li et al. 2007). In the basal state, GLUT4 storage vesicles (GSVs) are found in intracellular pools with GFP facing the cytoplasm (Figure 4B, left panel), and upon insulin stimulation vesicles fuse with the PM, causing exocytosis of 12-transmembrane GLUT4 channels. When translocated to the PM, the HA-GLUT4-GFP construct will have the C-terminal GFP positioned intracellular to the cell surface and the N-terminal HA tag positioned on the extracellular surface (Bogan, McKee, and Lodish 2001) (Figure 4B, right panel).

2. Adipocytes are an ideal cellular model to study GLUT4 translocation using HA-GLUT4-GFP chimera

In a non-permeabilizing immunofluorescence (IF) assay performed by the Zhao Lab using HA-GLUT4-GFP transfected in adipocytes (Zhao et al. 2009), the GFP fusion protein on GLUT4 was visualized without the use of a secondary fluorescent antibody, and it identified the intracellular distribution of exogenously expressed GLUT4. Upon insulin stimulation, once the GLUT4 protein translocated to the PM, the HA-epitope tag

was accessible to a secondary fluorescent antibody, which allowed visualization of GLUT4 along the cell surface by confocal immunofluorescence microscopy (Figure 4C). Thus, in starved conditions only the intracellular HA-GLUT4-GFP protein pool is observed, with GFP (green), because HA-GLUT4-GFP is not translocated to the PM and therefore the HA epitope is not detected by anti-HA antibody (Figure 4C, top panels). In insulin-stimulated cells, however, HA-GLUT4-GFP translocates to the PM and the HA signal along the PM can be detected (Figure 4C, bottom panels).

3. Adaptation of the HA-GLUT4-GFP translocation assay in HeLa cells

When we set out to study the role of GIV in GLUT4 translocation in adipocytes using the chimeric GLUT4 construct, we noted that differentiated adipocytes do not express full length GIV protein (*not shown*). This finding made the adipocytes an unsuitable model for studying GIV's role in GLUT4 trafficking. Because fatty acid-induced IR is a phenomenon that occurs in the skeletal muscle cells, and because GIV is expressed in skeletal muscles we rationalized that we will carry out initial sets of experiments in HeLa cervical carcinoma cells, which have been extensively used by multiple groups to elucidate mechanisms of IR in skeletal muscles. The second reason is that multiple groups had extensively studied GLUT4 translocation in these cells using the very same chemeric HA-GLUT4-GFP construct. First and foremost we wanted to successfully adapt the GLUT4 translocation assay to HeLa cells in our hands. We transformed and purified HA-GLUT4-GFP plasmid and verified transfection efficacy in parental HeLa cells, after 24 hrs and 36 hrs, under a fluorescent microscope (Figure 5A). GFP signal is first detected at 24 hrs (Figure 5A, left panel), and its signal is more than doubled following 36 hrs of transfection (Figure 5A, right panel). These results prove that

HeLa cells can be successfully transfected with HA-GLUT4-GFP and that the GFP signal is easily detected.

Next we wanted to optimize a nonpermeabilizing IF protocol in HeLa cells using the HA-GLUT4-GFP construct, just as had been done previously by multiple groups (Haga, Ishii, and Suzuki 2011; Li et al. 2007). For this aim, we performed and optimized an IF assay in which HeLa cells were transfected with HA-GLUT4-GFP for 36 hrs and either starved or insulin stimulated, and fixed under non-permeabilizing conditions. Cells were starved for 3 hrs in Krebs Ringer Bicarbonate (KRB) buffer (Haga, Ishii, and Suzuki 2011) and stimulated for 30 minutes with insulin, fixed and blocked, and then stained with anti-HA antibody for detection of HA signal on the extracellular surface of the PM. In starved HeLa cells we detected concentrated GFP signal at the perinuclear region (Figure 5B, top left panel) but no HA signal was found (Figure 5B, top middle panel). Upon insulin stimulation, HeLa cells showed an increase in the distribution of intracellular GFP (Figure 5B, bottom left panel) and a marked increase in HA signal along the PM (Figure 5B, bottom middle panel). Merged images of GFP and HA signals are also shown (Figure 5B, top and bottom right panels). These results substantiated that upon insulin stimulation, HA-GLUT4-GFP is translocated from an intracellular pool to the PM, thereby allowing the HA-tag to be accessible for detection using an anti-HA antibody, and we concluded that the approach adapted from adipocytes was successful. Furthermore, the low to none detection of HA signal intracellularly in the starved condition, reproduced in our cell model, demonstrated that we managed to optimize a non-permeabilizing IF protocol.

4. GIV is required for GLUT4 translocation

Because previous research has demonstrated GIV's critical role in activation of the PI3K-Akt pathway and linking activation of Akt to rapid actin remodeling (Anai et al. 2005; Enomoto et al. 2005; Garcia-Marcos, Ghosh, and Farquhar 2009), and because it has been well-established that the PI3K-Akt pathway is required for GLUT4 translocation to the PM (Martin et al. 1996), we asked whether GIV is required for GLUT4 translocation to the PM. To test this, we depleted HeLa cells of endogenous GIV using GIV siRNA (siGIV), and additionally we transfected these cells with HA-GLUT4-GFP and observed GLUT4 translocation in starved and insulin stimulated conditions. First, we measured the efficiency of GIV knockdown by immunoblot (IB) (Figure 6A) and found a dramatic decrease in the abundance of full length GIV protein (around 250 kDa) in siGIV-treated cells (Figure 6A, lane 2), as compared to those cells treated with control siRNA (siC) (Figure 6A, lane 1). We quantified the efficacy of GIV depletion by comparing the amount of GIV normalized to the loading control, tubulin, by band densitometry using Licor Odyssey, and found that GIV levels were decreased by ~70% in siGIV-treated cells (Figure 6B).

Next, we examined HA-GLUT4-GFP translocation to the PM in siC- versus siGIV-treated HeLa cells in starved and insulin-stimulated conditions (Figure 6C). To quantify this process, we counted within transfected cells the number of cells displaying GLUT4 translocation (identified by the presence of HA signal at the PM). An average of 100 transfected cells were counted per condition, and we found that both the siC- and siGIV-treated cells in the starved condition showed little to no GLUT4 translocation. In the insulin-stimulated condition, siC-treated cells displayed 63% GLUT4 translocation to

the PM as compared to only 19% translocation in siGIV-treated cells, approximately a 3-fold decrease. These results demonstrate that GLUT4 translocation, following insulin-stimulation, is downregulated in cells depleted of endogenous GIV, indicating that GIV is required for insulin-stimulated translocation of GLUT4 to the PM.

5. GIV's GEF function is required for GLUT4 translocation to the PM after insulin stimulation.

Next, we asked if GIV's GEF motif is required for GLUT4 translocation. To test this we generated HeLa cell lines stably expressing wild type GIV (HeLa-GIV-WT) and GIV-FA (HeLa-GIV-FA) and assessed percent of GLUT4 translocation to the PM by a non-permeabilizing immunofluorescence assay before and after insulin stimulation (Figure 7B). Based on quantification of 3 separate experiments (counting ~100 transfected cells per condition), we found that a low proportion (~ 20%) of GIV-WT and GIV-FA cells displayed GLUT4 translocation to the PM under starved conditions. Upon insulin stimulation GIV-WT cells, but not GIV-FA, displayed a dramatic increase in the proportion of cells that had successfully translocated GLUT4 to the PM: ~90% in the case of HeLa-GIV-WT and only around 30% in the case of GIV-FA. This difference was statistically significant (P-value of 0.0104; below 0.05 threshold) indicating that GIV's GEF function is required for GLUT4 translocation.

6. GIV's GEF function regulates key components of the insulin signaling pathway

Next we asked how GIV's GEF function may affect GLUT4 translocation. As previously mentioned, the InsR-IRS1-PI3K-Akt signaling axis has previously been demonstrated as a prerequisite for translocation of GLUT4 to the PM (Figure 2) (Saltiel and Kahn 2001). Because the extent of phosphorylation at Tyrosine 632 (Tyr 632)

(Esposito et al. 2001) of IRS-1 and at Serine 473 (Ser 473) (Alessi et al. 1996) for Akt have previously been used as readouts to assess the activation of this signaling axis, we analyzed the phosphorylation of these two proteins in HeLa stable cell lines (GIV-WT and GIV-FA) before and 5 min after stimulation with insulin as done previously (Garcia-Marcos et al. 2011) by immunoblotting. We found that phosphorylation levels of IRS-1 and Akt were significant higher in GIV-WT cells when compared to GIV-FA cells (Figure 8A). The decrease in activating phosphorylations of IRS-1 and Akt we observe in GIV-FA cells indicate that activation of these components in the insulin signaling pathway rely significantly on a functional GIV's GEF motif.

7. Phosphoinhibition of GIV-GEF by PKCθ results in suppression of GLUT4 translocation and IRS-1 activation

Because PKCθ causes IR, and phosphorylation of GIV by this kinase inhibits GIV's GEF activity, next we asked if phosphoinhibition of GIV's GEF motif by PKCθ can result in altered insulin signaling or suppression of GLUT4 translocation as seen in IR states. To study this we took advantage of a previously established HeLa cell line stably expressing the S1689D GIV mutant, a biological mimetic of phosphoinhibition at S1689 by PKCθ (Lopez-Sanchez et al. 2013). We found that the phenotype of GIV-SD cells very closely matched that of GIV-FA cells (Figure 7A, B), in which there were ~20% cells with GLUT4 translocation to the PM in the starved condition and only ~30% of cells with GLUT4 translocation to the PM in the stimulated condition. The P-value for GIV-SD was 0.0214, compared to GIV-WT, which also verified the significance of our data (Figure 7B). We concluded that phosphoinhibition of GIV's GEF function by PKCθ inhibits GLUT4 translocation. In addition, when we analyzed the signaling program in

these HeLa-GIV-SD cells (as outlined for Figure 8 in the section above) we found that as in the case of the GEF-deficient HeLa-GIV-FA cells activation of IRS1 and Akt in these cells was blunted (Figure 8, lane 6) when compared to HeLa parental or GIV-WT cells that expressed a functional GIV's GEF domain (Figure 8, lanes 2 and 4). From these results we conclude that PKCθ phosphoinhibits GIV's GEF domain and thus interferes with activation of IRS-1 downstream of the InsR.

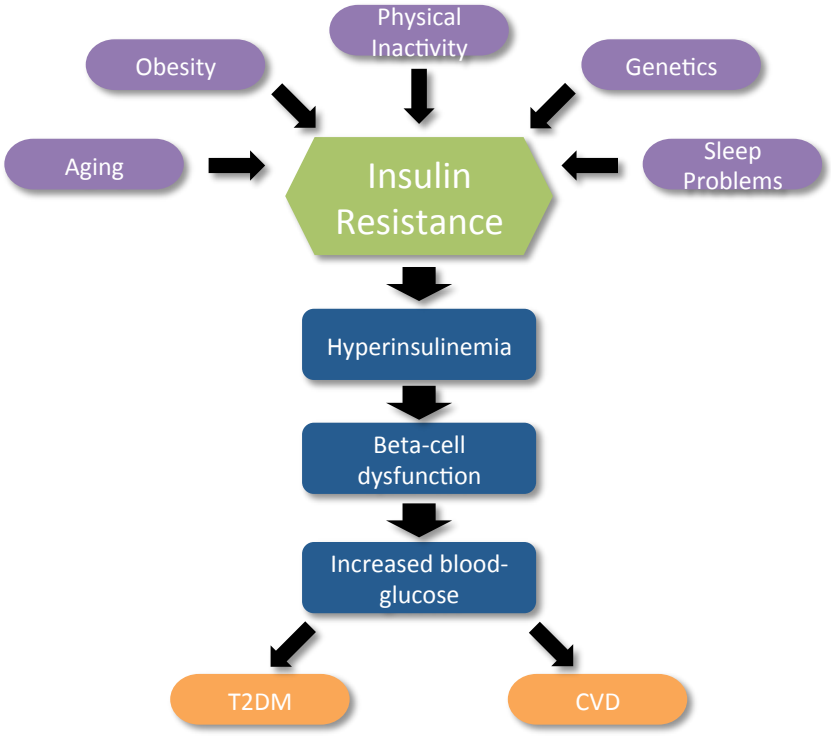

Figure 1. Causes and effects of insulin resistance (IR). Risk factors for developing IR include aging, obesity, sedentary lifestyle, genetics, and sleep problems such as sleep apnea. Insulin resistance results from reduced sensitivity of cells to insulin, therefore promoting the pancreatic islet beta cells to secrete excess amounts of insulin (hyperinsulinemia). Beta cells will eventually fail to keep up with the body's continuous demand for insulin, and an increased blood glucose level (hyperglycemia) will set the stage for development of T2DM, among other systemic diseases.

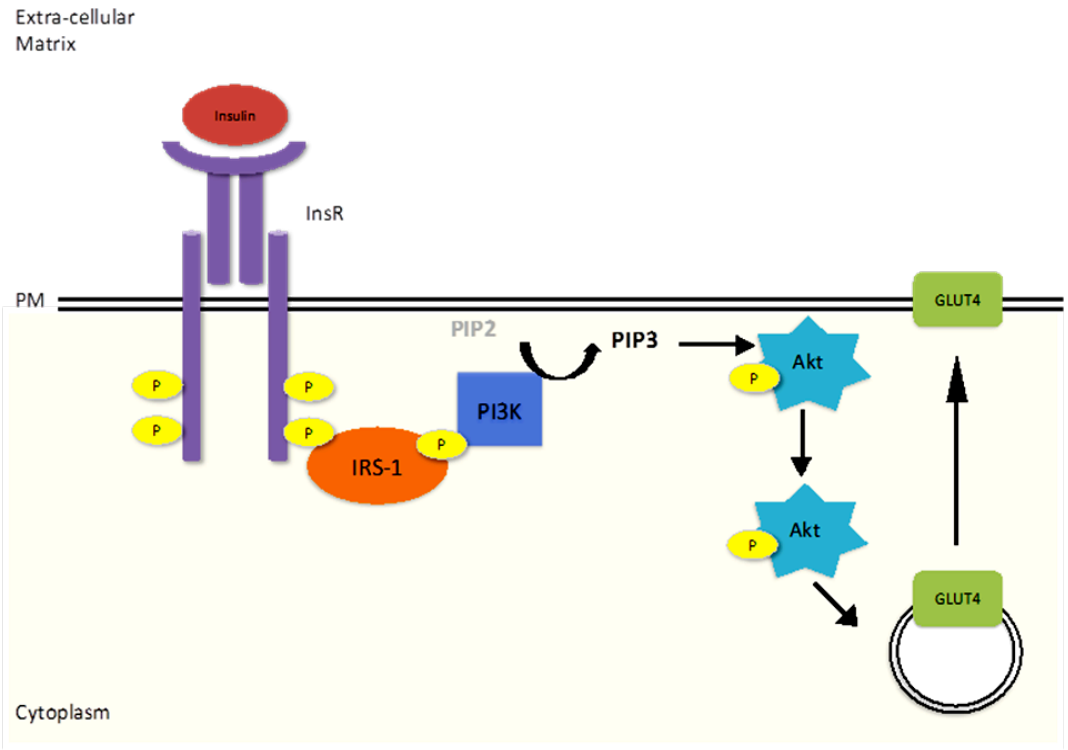

Figure 2. Activation of the InsR→IRS1→PI3K→Akt axis of signaling is required for rapid translocation of GLUT4 to the PM. Upon binding of hormone insulin to its receptor, InsR, cytoplasmic tails on the receptor are autophosphorylated at tyrosine residues, and will recruit and phosphorylate IRS-1. Activated IRS-1 will then phosphorylate and activate PI3K, which will convert membrane-bound PIP2 to PIP3, thus leading to phosphorylation of Akt. Activated Akt promotes translocation and fusion of GLUT4 transmembrane channels from cytosolic vesicles to the PM.

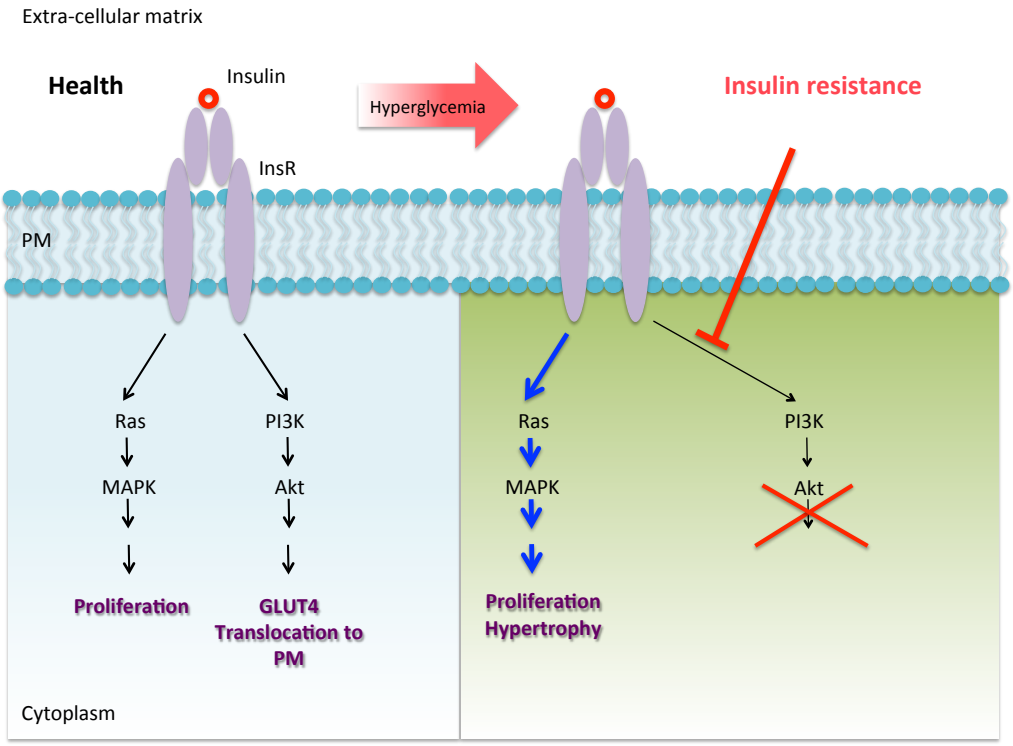

Figure 3. Insulin sensitivity requires balanced signaling between the PI3K-Akt and the MAPK-ERK signaling pathways; tilting this balance heralds IR. In physiology (left panel), insulin sensitivity is maintained by two separate yet balanced signaling pathways that are triggered downstream of an activated InsR: the PI3K-Akt and MAPK-ERK pathways. While the MAPK-ERK pathway is responsible for growth/mitogenic effects of insulin hormone, the PI3K-Akt pathway is largely implicated in mediating the melabolic effects of the hormone. Under conditions of IR (right panel), this balance is tilted in favor of the MAPK-ERK pathway, with an upregulation of MAPK-ERK and a simultaneous downregulation of the PI3K-Akt pathway.

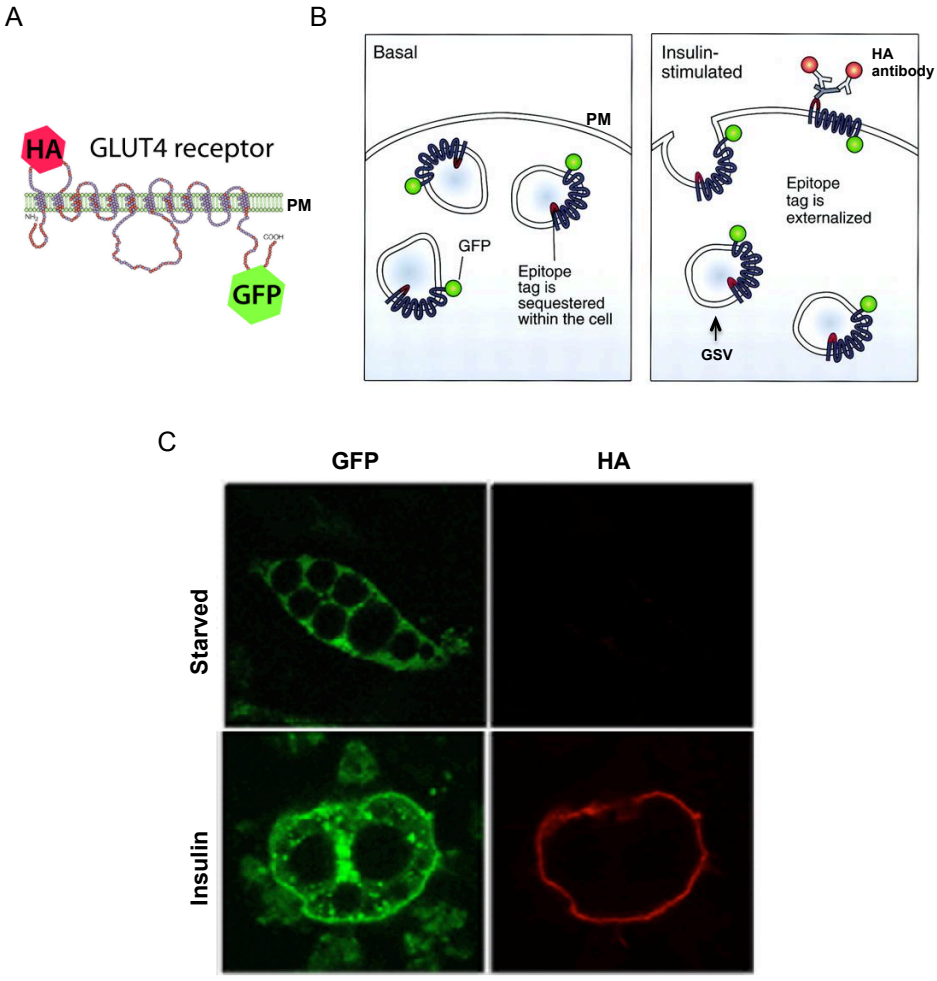

Figure 4. HA-GLUT4-GFP construct. (A) Illustration depicting GLUT4 protein with HA epitope tag on the N-terminal exofacial loop, positioned extracellular to the PM, and GFP tag fused to the C-terminus, positioned on the cytosolic side. (B) Illustration depicting position of HA-GLUT4-GFP in GLUT4 storage vesicles (GSVs) in the basal state of a cell (left panel) and during fusion and exocytosis of HA-GLUT4-GFP with the PM after insulin-stimulation (right panel). GFP tag (green) remains cytosolic and HA tag (red) faces extracellular surface of cell. (C) Adipocytes transfected with HA-GLUT4-GFP, in starved (top panels) and insulin-stimulated (bottom panels) conditions, processed by IF for anti-HA antibody. GFP (green signal) is shown on left and HA (red signal) is shown on right. Figure modified from: *Variations in the requirement for v-SNAREs in GLUT4 trafficking in adipocytes,* (Zhao et al. 2009).

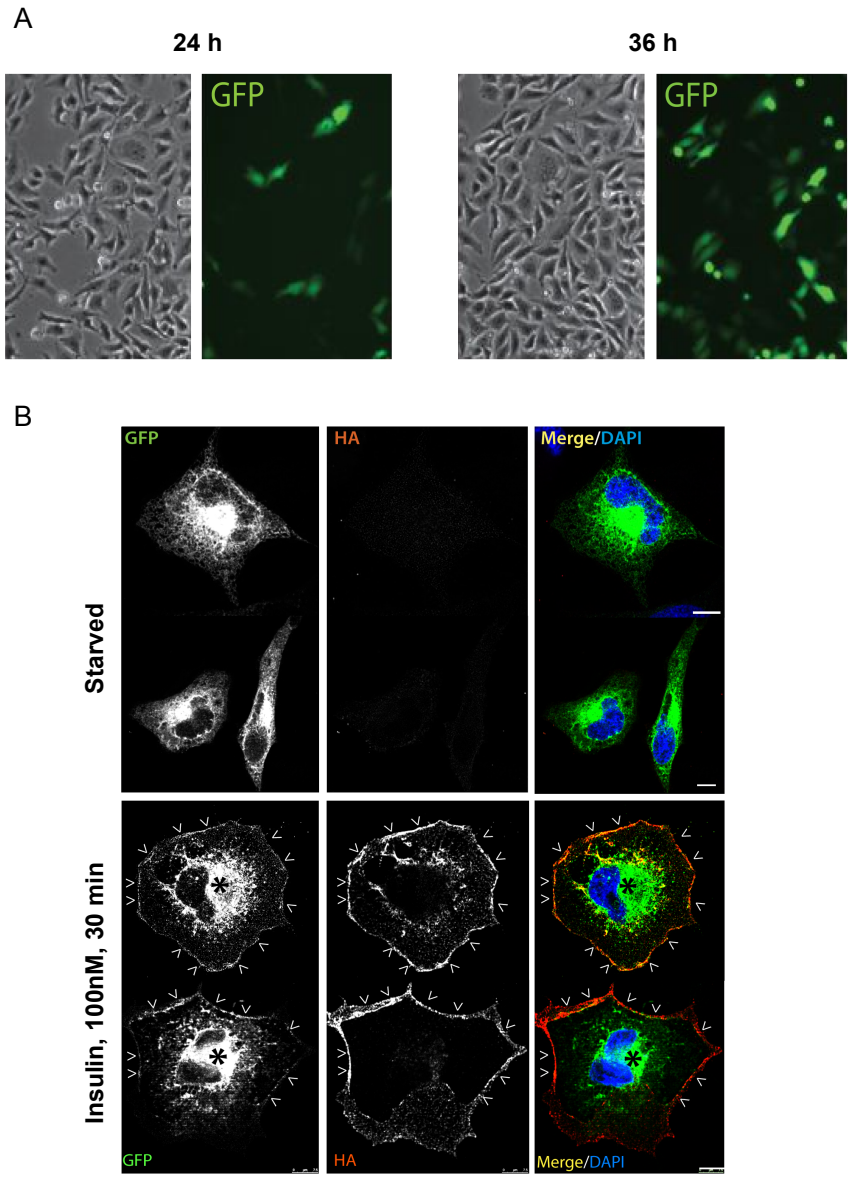

Figure 5. HA-GLUT4-GFP translocation in HeLa cells. (A) HeLa cells transfected with 1 µg HA-GLUT4-GFP plasmid show expression of GFP at 24 and 36 hrs, showing increasing intensity with time from left panel to right panel. (B) HeLa cells transfected with HA-GLUT4-GFP as above were serum starved for 3 hrs in Krebs Ringer Bicarbonate (KRB) buffer and then treated with Insulin. Fixed cells were stained for HA (red) and with DAPI (DNA, blue). Scale bars, 10 µM.

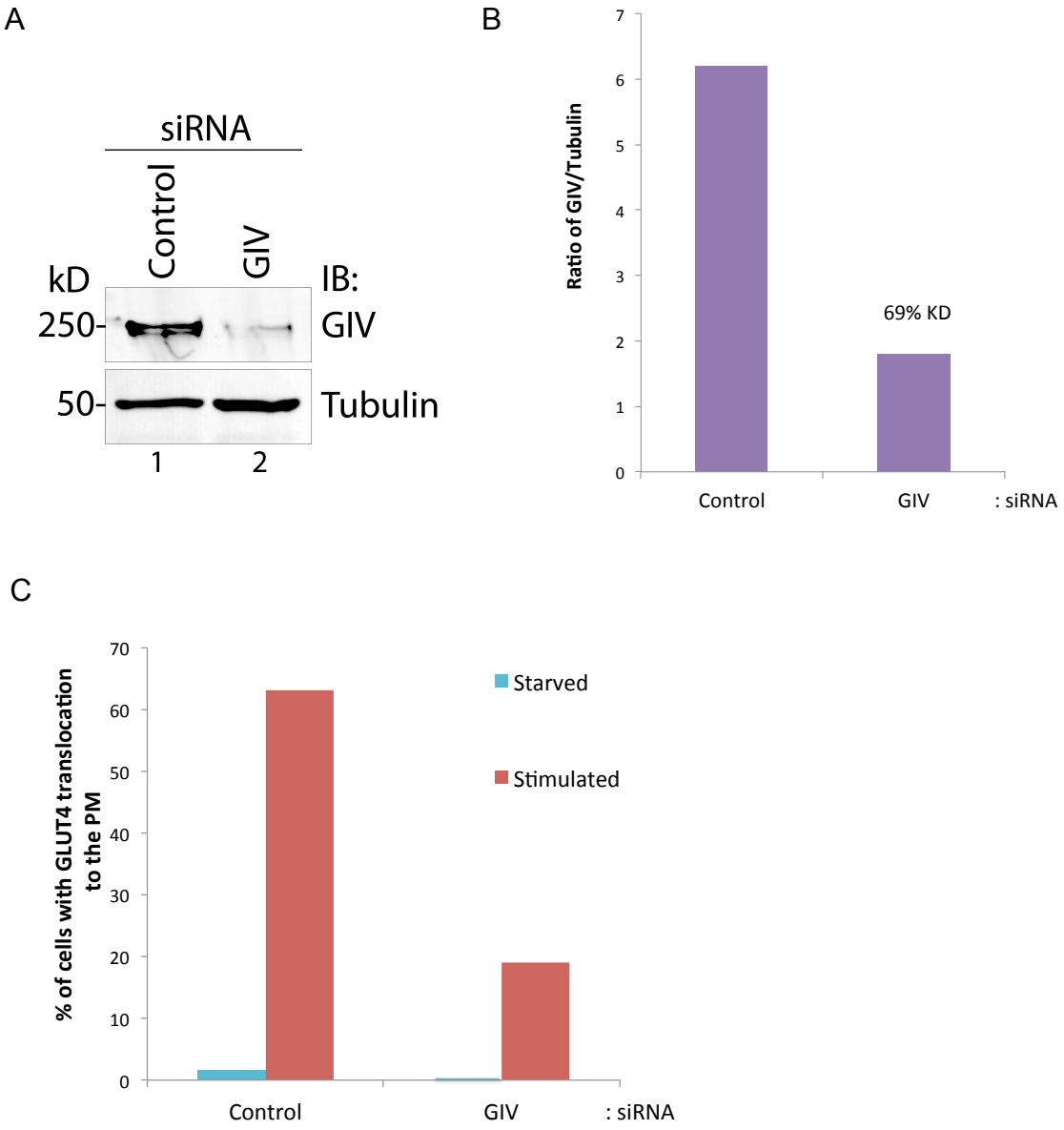

Figure 6. Depletion of GIV by siRNA inhibits insulin-stimulated translocation of GLUT4. (A) HeLa cells were transiently transfected with control or GIV siRNA and whole cell lysates were analyzed for GIV and tubulin by IB. (B) Percent GIV depletion in Figure A was estimated by band densitometry analysis and normalizing to loading control tubulin by LiCOR Odyssey. Quantification is shown as a bar graph. (C) HeLa cells transiently transfected with HA-GLUT4-GFP and either control or GIV siRNA were serum starved for 3 hrs in KRB buffer and then treated with 100 nM insulin, 30 min. Cells were stained for HA (red) and DAPI (blue), and approximately 100 transfected cells were counted per condition to analyze how many cells had GLUT4 translocation. Quantification is shown in a bar graph, with the % of GLUT4 translocation to the PM displayed on the y-axis.

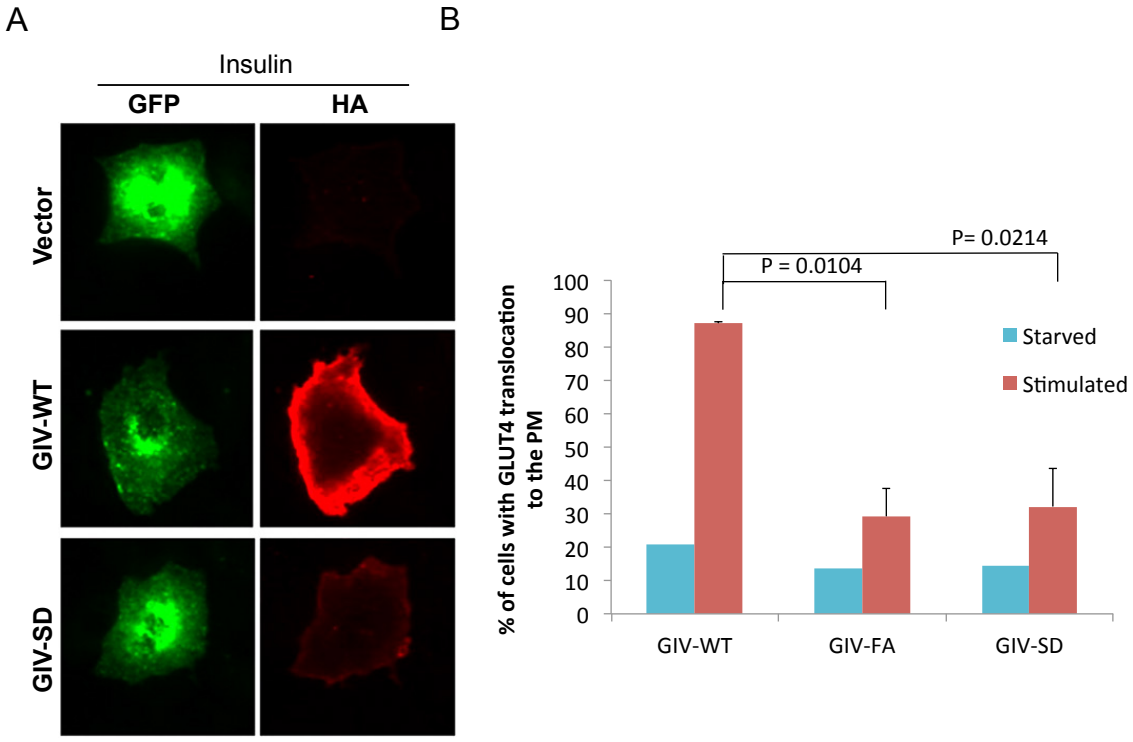

Figure 7. Insulin-stimulated translocation of GLUT4 to the PM is impaired in GIV-FA and SD cells when compared to HeLa-GIV-WT cells. (A) HeLa cells stably expressing GIV-WT or GIV-SD were transfected with HA-GLUT4-GFP plasmid, serum starved for 3 hrs in KRB buffer and stimulated with 100 nM insulin, 30 min. Cells were stained for HA (red). (B) For HeLa cells expressing GIV-WT, GIV-FA, or GIV-SD in starved or insulin-stimulated conditions, approximately 100 transfected cells were counted per condition. Quantification is shown in a bar graph, with the % of GLUT4 translocation to the PM displayed on the y-axis, and P-values for GIV-FA and GIV-SD compared to GIV-WT calculated; n=2 experiments.

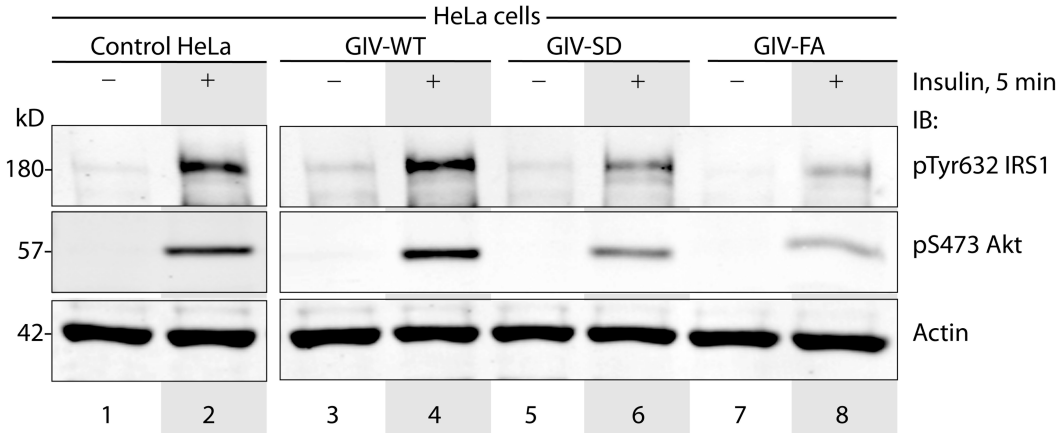

Figure 8. Upon insulin stimulation, phosphorylation of IRS-1 and Akt are impaired in Hela-GIV-FA and GIV-SD cells when compared to HeLa-GIV-WT cells. Parental HeLa and HeLa cells stably expressing GIV-WT, GIV-FA, or GIV-SD were serum starved for 3 hrs in KRB buffer and stimulated with 100 nM insulin, 5 min. Whole cell lysates were immunoblotted for pTyr 632 IRS-1 (activation site), phosho-Ser 473 Akt (activation site) and actin. In stimulated conditions, when we compared HeLa parental cells used as control (lanes 1 and 2) to cells expressing GIV-WT, we saw an increase in the phosphorylation and activation of IRS-1 and Akt in GIV-WT cells (lane 4). However, there was a decrease in these phosphorylation signals in both the GIV-SD and GIV-FA mutant cell lines (lanes 6 and 8).

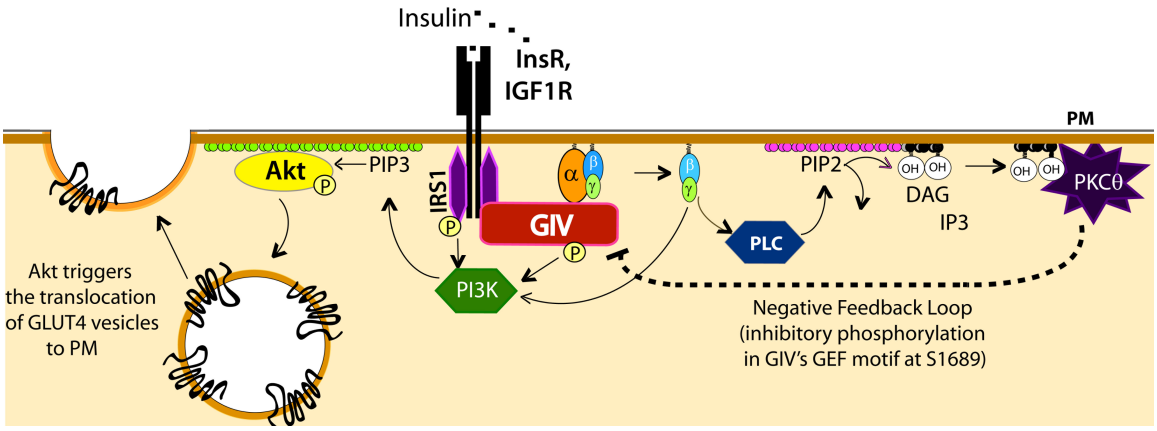

Figure 9. Our working model for how GIV's GEF function regulates insulin response and how phosphoinhibition of GIV's GEF function can induce IR. Upon insulin stimulation, InsR undergoes autophosphorylation at multiple sites on its cytoplasmic tail. GIV and IRS1 are recruited to the ligand-activated InsR, by directly binding to these autophosphorylation sites. GIV's GEF motif mediates further recruitment of Gi to ligand- stimulated InsR. Subsequent activation of the PI3K-Akt pathway happens by several independent and parallel mechanisms: a) Activation of Gi by GIV-GEF in the vicinity of activated receptors releases 'free' Gβγ, which in turn can activate Class 1b PI3Ks (Garcia-Marcos, Ghosh, and Farquhar 2009) b) Recruited GIV and IRS1 also undergo activation by tyrosine phosphorylation and directly bind and activate Class 1a PI3Ks (Lin et al. 2011; Saltiel and Kahn 2001). Presence of GIV (alongside IRS1) in the vicinity of the InsR causes increased recruitment of PI3K to the receptor (Lin et al. 2011). Activated PI3K-Akt pathway triggers translocation of GLUT4 channels from storage vesicles to the plasma membrane (PM). G protein intermediates can trigger activation of PKCθ, which phosphorylates GIV at S1689, thus inhibiting its GEF function and downregulating the PI3K-Akt pathway, which leads to a decrease in the translocation of GLUT4 channels to the PM. We propose that circulating fatty acids that trigger IR via activation of PKCθ, actually do so in part by phosphoinhibition of GIV-GEF.

III:
Discussion

GIV is a key mediator of insulin response whose GEF function is required for efficient translocation of GLUT4 vesicles to the PM

We demonstrated that in HeLa cells depleted of endogenous GIV, the translocation of GLUT4 to the PM substantially decreased by approximately 70%, suggesting that GIV is involved in regulating GLUT4 translocation. Similarly, in GEF-mutants cell lines, GIV-FA and GIV-SD, there was nearly a 60% decrease in GLUT4 translocation compared to GIV-WT cells in insulin-stimulated conditions. Previous findings have established GIV, and more specifically GIV's GEF motif, as a regulator of PI3K-Akt signals (Anai et al. 2005; Enomoto et al. 2005; Enomoto, Ping, and Takahashi 2006; Garcia-Marcos, Ghosh, and Farquhar 2009), and separately, Akt has been established as a regulator of GLUT4 translocation to the PM (Saltiel and Kahn 2001). Therefore, we propose that GIV's GEF function is integral to the regulation of insulin-stimulated GLUT4 trafficking, a cellular function that is dysregulated in IR and T2DM.

To study GIV's role in modulating GLUT4 translocation to the PM, it is important to first understand the step-by-step mechanism of GLUT4 trafficking. Insulin stimulated GLUT4 trafficking involves a downstream signaling cascade from the InsR, which results in the release of GLUT4 storage vesicles (GSVs) from intracellular retention, translocation of GSVs along cytoskeletal structures, and finally tethering, docking, and fusion of GSVs at the PM (Rowland, Fazakerley, and James 2011). We already know that GIV regulates the PI3K-Akt pathway, a critical signaling step downstream of the InsR. Therefore, of the trafficking events mentioned above, we believe that GIV could be involved in release of GSVs from intracellular retention

(indirectly by regulating the components of downstream insulin signaling), their translocation through the cytoskeleton, and tethering at the PM.

Translocation of GSVs begins with their release from intracellular retention (Rowland, Fazakerley, and James 2011). Insulin stimulation triggers a signaling cascade that activates IRS-1, which then activates the PI3K-Akt pathway and thus promotes GLUT4 translocation (Saltiel and Kahn 2001). In this project, we demonstrated that following insulin stimulation, cells overexpressing GIV-WT exhibited a greater amount of activated IRS-1 (identified by phosphorylation at a critical residue, Tyr632) compared to the GIV-FA and GIV-SD mutants, thus demonstrating GIV GEF function as a regulator of IRS-1. Separate findings have already demonstrated that GIV is a regulator of PI3K-Akt activation by two separate yet synergistic mechanisms: phosphorylation at critical tyrosine residues at the C-terminus of GIV allows for GIV-PI3K complexes to form and enhancement of Akt activation, and GIV's GEF motif binds and activates Gαi3, resulting in a release of the Gßγ subunits that then bind and activate PI3K (Enomoto, Ping, and Takahashi 2006; Garcia-Marcos et al. 2010; Lin et al. 2011). Other studies on the role of Akt have found that upon insulin stimulation, it promotes translocation of GSVs to the PM. Activated Akt phosphorylates TBC1D4, a Rab GTPase activating protein (Rab-GAP), and thus inhibits its' GAP function. Inactive TBC1D4 can no longer act on its substrate, Rab, a GTPase associated with GSVs, therefore allowing GTP loading on Rab and ultimately resulting in GSV translocation (Rowland, Fazakerley, and James 2011). We propose that since GIV is a regulator of Akt, and other research findings have proven that Akt triggers GLUT4 translocation, it is possible that GIV regulates GLUT4 translocation via its role as a regulator of the PI3K-Akt pathway,

although more research is required to further develop this hypothesis. An interesting project would be to study if GIV's GEF-mutant cell lines will exhibit a decrease in GTP-loading on Rab proteins associated with GSVs.

Following their release, GSVs move through the cytoskeletal matrix of a cell by actin remodeling (Rowland, Fazakerley, and James 2011). Since previous findings have identified Akt-dependent GIV as a regulator of actin remodeling at the leading edge of migrating cells upon growth factor stimulation (Enomoto et al. 2005; Enomoto, Ping, and Takahashi 2006), it is plausible that GIV assists in the translocation of GSVs through the cytoskeleton. Once GSVs have reached the PM, the initial step in GLUT4 translocation at the cell surface involves tethering, a step that ensures specificity of the fusion reaction prior to vesicle fusion. Research findings have shown that this initial step is aided by exocyst complexes, which consist of 8 exocyst subunits (Larance, Ramm, and James 2008). In a recent study by Camargo *et al*, yeast two-hybrid screens suggested an interaction between GIV and Sec3, an exocyst subunit (Camargo et al. 2007), although this remains yet to be proven. Therefore, it is possible that GIV interacts with exocyst complexes near the PM during GLUT4 translocation and tethering. In keeping with what we know about GIV and its regulation of actin remodeling and possible interaction with Sec3, we propose that GIV could assist the translocation of GSVs through the cytoskeleton and tethering of GSVs to the PM. In the future, it would be interesting to identify if GIV directly interacts with GSVs during their translocation through the cytoskeletal matrix, if GIV interacts directly with Sec3 exocyst complexes, and if the GIV-Sec3 interaction can modulate GSV fusion at the PM.

GIV's GEF function maintains insulin sensitivity by enhancing the activation of IRS-1 and Akt

We already discussed GIV's role in insulin signaling via regulation of PI3K-Akt, and our results from this project identified GIV's ability to regulate another key component of insulin signaling, which is phosphorylation of IRS-1. Since activation of IRS-1 occurs upstream of the PI3K-Akt pathway, it is possible that GIV regulates multiple steps in insulin signaling, yet it remains unknown whether this level of regulation is directly influencing GLUT4 translocation.

Research findings have identified IRS-1 as a regulator of both the PI3K-Akt and the MAPK-ERK pathways (Cusi et al. 2000; Thirone, Huang, and Klip 2006; Yu, Gao, and Ma 2011). GIV was also recently identified as a regulator of these two signaling pathways; Garcia-Marcos *et al.* demonstrated that GIV-FA cells expressed a significant downregulation in levels of phosphorylated Akt and a significant upregulation in levels of phosphorylated ERK compared to GIV-WT cells (Garcia-Marcos et al. 2011). This dichotomy of PI3K-Akt versus MAPK-ERK signaling patterns is in keeping with previous findings on the pattern differences observed in insulin responsive versus insulin resistant cells (Cusi et al. 2000; Thirone, Huang, and Klip 2006; Yu, Gao, and Ma 2011). Based on our findings that GIV regulates IRS-1 phosphorylation, and the findings from other groups on PI3K-Akt and MAPK-ERK phosphorylation patterns downstream of IRS-1 in insulin resistant conditions, we hypothesize that GIV is involved in GLUT4 translocation via its regulation of IRS-1 and PI3K-Akt. To further develop this hypothesis, we could test if GIV physically interacts with IRS-1 or if the regulation of

IRS-1 by GIV is indirect, and whether this interaction is absent in insulin-resistant patients.

Phosphoinhibition of GIV's GEF function by PKCθ at S1689 inhibits GLUT4 translocation and inhibits IRS-1

We demonstrated that disruption of GIV's GEF function by using GIV-SD mutant, previously established as a biological mimetic of phosphoinhibition by PKCθ at this S1689 residue (Lopez-Sanchez et al. 2013), resulted in downregulation of GLUT4 translocation to the PM. These results probed us to ask if this downregulation, mediated by suppression of GIV's GEF function, could by a direct result of dysregulation in IRS-1 or PI3K-Akt downstream of the InsR, and we found that phosphoinhibition of GIV's GEF by PKCθ resulted in suppression of IRS-1 activity (identified by phosphorylation at activatory site Tyr632) upon insulin-stimulation.

We already knew that GIV is required for facilitating interactions between InsR and heterotrimeric G protein Gi (Lin et al. Submitted (2013)), and that Gi proteins were required for PI3K-Akt mediated GLUT4 translocation downstream of the InsR (Ciaraldi and Maisel 1989), and we believed that this could be a second parallel pathway alongside IRS-1 that mediated insulin response. Although the exact mechanim by which GIV regulates phosphorylation of IRS-1 is not known, it is possible, as we speculated earlier, that when GIV binds InsR to mediate activation of Gi protein, it displaces other competing adaptors proteins at the cytoplasmic tails of InsR, such as IRS-1. This can be studied further by observing if there is binding between IRS-1 and GIV at the InsR simultaneously upon insulin stimulation, and how this might be altered in GEF mutant GIV cells lines.

Multiple groups have already established the role of PKCθ as a phosphoinhibitor of IRS-1 downstream of the InsR and a suppressor of PI3K-Akt-dependent GLUT4 translocation (Haasch et al. 2006; Kewalramani et al. 2011; Yu et al. 2002), orchestrating development of IR in muscle and adipose cells. However, research by Hoehn *et al.* in 2008 also found that IRS-1 alone was not enough to reverse IR in cells (Hoehn et al. 2008), and this pointed to a potential second molecular candidate on which PKCθ acted to suppress insulin-stimulated GLUT4 translocation and glucose uptake, and our results suggest that PKCθ triggers insulin resistance in part via phosphoinhibiting GIV.

Based on our results and what is known about GIV, InsR and PKCθ, we conclude that GIV facilitates the interaction between InsR, heterotrimeric G proteins, and PI3K to enhance PI3K-Akt signaling and GLUT4 translocation after insulin stimulation and subsequent glucose uptake. Phosphoinhibition of GIV by PKCθ has emerged as a novel mechanism for how fatty-acids induce IR. By serving as a key molecule for maintaining insulin sensitivity and as a major pathway for establishment of IR, GIV appears to be a promising target which can be manipulated to reinstate insulin sensitivity in cells that are resistant. Further mechanistic insights into how GIV and its GEF function may affect all other aspects of insulin response and GLUT4 translocation is both critical and urgent because such knowledge will help us define a novel target for drug development in the treatment of IR and Diabetes.

IV:

Materials and Methods

Heat shock transformation

50 ng of desired vector/DNA construct was added to 50 µl of competent DH5α cells. Eppendorf tubes containing competent cells and DNA were incubated on ice for 30 min, and then submitted to heat shock at 42^{o}C for 45 seconds. Then cells were incubated on ice for 2 min before adding 1 mL of fresh LB media with no antibiotics, and incubated at 37^{o}C for 1 hr on a shaker. From this culture, 100 µl was spread on LB plates with appropriate antibiotics and grown overnight.

DNA plasmid purification

Plasmids were purified using a HiPure Plasmid Filter Midiprep Kit from Invitrogen. To obtain DNA for our gene of interest, we picked one colony from the transformed LB plates and it was grown in 20 mL LB (with antibiotic) for 3-4 hrs, and then transferred into 500 mL LB (with antibiotic) and incubated overnight at 37^{o}C. Next day we proceeded with the Invitrogen Midiprep protocol.

DNA concentration measurement

DNA concentration was measured using Nanodrop Software (Thermo Scientific 2011).

Ponceau staining

Polyvinylidene fluoride (PVDF) membranes (Millipore), after transfer, were stained with Ponceau S Staining solution (Sigma Aldrich) for 20 minutes at room temperature. After this incubation, membranes were washed with water to remove non-specific staining and proteins were visualized in pink. After cutting the membranes at specific locations along the protein marker for immunoblotting (IB), membranes were washed with 1X PBS to remove the Ponceau S Stain.

Cell culture, ligand stimulation, and cell lysis

Parental and stable HeLa cell lines were cultured according to ATCC guidelines. They were grown in DMEM (1X, with 4.5 g/L glucose and L-glutamine without sodium pyruvate) containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin-Glutamine (P/S/G) antibiotic, and 500 µg/mL selection media G418 Sulfate solution in the case of the stable cell lines. Cells were passaged when they reached 100% confluency using ATV. Cells were grown in an open system incubator at 37^{o}C and 5% CO_2.

The stable cell lines used in this thesis were generated by transfection of GIV-3xFLAG wild-type (HeLa-GIV-WT), GIV-F1685A-3xFLAG mutant (HeLa-GIV-FA), or GIV-S1689D-3xFLAG mutant (HeLa-GIV-SD) followed by selection with 800µg/mL G418 during the initial 6 weeks, and maintained in 500 µg/mL G418 media. Constructs are described in detail in Table 3.

Unless otherwise indicated, in assays that involved serum starvation, stable and parental HeLa cell lines were serum-starved overnight in DMEM containing 0.2% FBS. Stimulation with 100 mM Regular Human Insulin (Recombinant DNA origin, Novolin) was used.

Unless otherwise specified, cells were lysed by whole cell lysis after washing cells two times with cold 1X PBS. Cells were resuspended in lysis buffer (see Table 2) and then boiled with an equal volume of 5X sample buffer for 5 min (see Table 2).

DNA transfection

DNA transfection was carried out using Genejuice reagent (Novagen) and following Novagen Company guidelines. First, we added 100 µl (per µg of DNA) of 1X

Opti-MEM (reduced serum medium, Gibco Life Technologies) and 3 µl of Genejuice transfection reagent, and the mix was incubated at room temperature for 5 min.

In most cases, unless specified differently, to the transfection mix we added 1 µg of HA-GLUT4-GFP DNA (Table 3) and incubated at room temperature for 20-25 min before transfecting HeLa cells. DNA transfection was carried out in 36-48 hrs.

siRNA transfection

Invitrogen transfection guidelines were followed for siRNA transfection in HeLa cell lines. Cells were 50-60% confluent at the time of transfection. In polystyrene tubes we added Opti-MEM (100 µl/well for a 9.6 cm^2 plate) and scrambled or GIV siRNA oligos (1 µl of 75µM stock). In eppendorf tubes we added 100 µl/well of Opti-MEM and 2 µl Oligofectamine Reagent (Invitrogen) (1:2 ratio of siRNA: Oligofectamine). The solutions were incubated at room temperature for 5 min. Then the siRNA/Opti-MEM mix was added drop-by-drop to the Oligofectamine/Opti-MEM mix and incubated for 20-25 min at room temperature. Cells were washed with Opti-MEM 2x and left in 800 µl Opti-MEM before adding 200 µl of transfection mix. Cells were incubated for 5 hrs at 37^oC and then we added 1 mL of 30% FBS media. Cells were incubated overnight and then switched to fresh 10% FBS media, and optimal transfection was reached at 48 hrs.

Immunoblot

Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in presence of 1X Running Buffer (Table 2) at 90-120V for approximately 1.5 hrs or until the proteins of interest were properly separated using a protein marker as reference (Precision Plus Protein All Blue Standard, Bio-Rad Laboratories, Inc.). Proteins from gel were then transferred to PVDF membranes in

presence of 1X Transfer Buffer (Table 2) at 120V for 2 hrs or 35V overnight. Membranes were blocked in 1X PBS supplemented with either 5% BSA (Sigma) for phosphorylated proteins or with 5% dry nonfat milk for non-phosphorylated proteins. Membranes were then incubated overnight in primary antibodies, washed in 1X PBS-T (1X PBS with 1:1000 dilution Tween-20) and incubated in secondary fluorescent antibodies (see Table 1). After secondary incubation, membranes were washed again 2x in PBS-T and a third wash in 1X PBS. We used Li-Cor Odyssey imaging system to detect and quantify two-color infrared imaging. We used Image J software (National Institute of Health) to process and assemble Odyssey images.

Immunofluorescence of non-permeabilized cells

Cells were fixed with 3% paraformaldehyde (diluted in 1X PBS) for 10 min on ice followed by 15 min at room temperature, and then incubated in 100 mM Glycine (diluted in 1X PBS) to neutralize aldehyde groups. Fixed cells were then incubated in non-permeabilizing Blocking Buffer (Table 2) overnight, and incubated in primary antibody (diluted in non-permeabilizing blocking buffer) for 1hr at room temperature. Cells were washed 3x for 10 min in 1X PBS, incubated in secondary fluorescent antibodies at 1:500 dilution (Table 1) and DAPI at 1:1000 dilution (Molecular Probes, Invitrogen) for 1 hr, washed 3x for 10 min, and placed on cover slides over 5-6 µl of ProLong Gold antifade reagent (Invitrogen). Images were acquired with a Leica CTR4000 Confocal Microscope using a 63x objective. All images were processed using Image J-software.

Statistical analysis

Each experiment presented in these figures represents a minimum of three independent experiments. Statistical significance (P-value) was assessed using a t-test on Microsoft excel, and values remaining at $P<0.05$ were considered statistically significant. Standard deviations were also calculated using Microsoft excel.

Table 1. Antibodies

Name	Isotype	Antigen	Dilution	Company
p-IRS-1 (Tyr 632)	Rabbit polyclonal IgG	Short aa sequence around phosphorylated Tyr 632 of IRS-1	IB 1:500	Santa Cruz Biotech
p-IRS-1/2 (Tyr 612)-R	Rabbit polyclonal IgG	Short aa sequence around phosphorylated Tyr 612 of IRS-1	IB 1:500	Santa Cruz Biotech
Insulin Rß (C-4)	Mouse monoclonal IgG_{2a}	aa 941-1010 human origin	IB 1:250	Santa Cruz Biotech
p-Akt (Ser473)	Rabbit polyclonal IgG	Short aa sequence around Ser473	IB 1:250	Cell Signaling
p-Akt (Thr308) (C31E5E)	Rabbit polyclonal IgG	Short aa sequence around Thr473	IB 1:250	Cell Signaling
GFP (B-2)	Mouse monoclonal IgG_{2a}	aa 1-238 full length GFP	IB 1:500	Santa Cruz Biotech
Pospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit monoclonal IgG	Synthetic phosphopeptide (KLH-coupled)	IB 1:250	Cell Signaling
ß Tubulin (AA2)	Mouse monoclonal IgG_1	aa 412-430	1:500	Santa Cruz Biotech
ß-Actin	Mouse monoclonal IgG_{2a}	N-terminal peptide cytoplasmic ß-Actin	IB 1:1000	Sigma Aldrich
HA.11	Mouse monoclonal IgG_1	twelve amino acid peptide CYPYDVPDYASL	IB 1:500 IF 1:1000	Covance
Anti-Girdin C terminus (GIV-CTAb) (T-13)	Rabbit polyclonal IgG	Carboxy terminus Girdin	IB 1:500	Santa Cruz Biotech
Anti-Girdin GIV #14	Rabbit polyclonal IgG	Coiled Coil domain	IB 1:500	Millipore
Alexa Fluor 680 (Odyssey)	Rabbit IgG	F(ab')2 fragments	IB 1:10,000	Invitrogen Life Technologies
Alexa Fluor 800 (Odyssey)	Mouse IgG	F(ab')2 fragments	IB 1:10,000	Invitrogen Life Technologies
Alexa Fluor 594	Mouse monoclonal IgG	F(ab')2 fragments	IF 1:500	Invitrogen Life Technologies
Alexa Fluor 594	Rabbit polyclonal IgG	F(ab')2 fragments	IF 1:500	Invitrogen Life Technologies

Table 2. Buffers

Name	Components	Preparation	Company
Krebs-Ringer Bicarbonate Buffer, pH=7.4 (with 1.8g/L glucose, w/o $CaCl_2$ and $NaHCO_3$)	1.8 g/L D-glucose, 0.047 g/L $MgCl_2$, 0.34 g/L KCl, 7.0 g/L NaCl, 0.1 g/L Sodium Phosphate Dibasic [anhydrous] and 0.18 g/L Sodium Phosphate Monobasic [anhydrous].	Add powdered medium to water at 15-20 ^oC, add 1.26 g $NaHCO_3$ for each liter of final volume. Bring pH to 7.4 and sterilize immediately by filtration using 0.22 micron porous membrane	Sigma-Aldrich (Haga, Ishii, and Suzuki 2011)
Lysis Buffer	20 mM Hepes pH 7.2, 125 mM K-acetate and 5 mM Mg-acetate	To 10 ml buffer, add 2 mM DTT, 1 tablet of Roche Protease inhibitor cocktail (Complete, EDTA-free), 0.4 % Triton X-100, 1X phosphatase inhibitor cocktail 2 and 3, 0.2 mM Sodium Orthovanadate	Made in the Lab
10X Running Buffer	0.25 M Tris, 1.92 M Glycine, 1% SDS, H_2O	Add 1 mL of 10% SDS to 1 L of 1X Running Buffer	
10X Transfer Buffer	0.2M Tris, 1.29 M Glycine, H_2O		
5X Sample Buffer	5% SDS, 156 mM Tris, 25% glycerol, 0.025% Bromophenol Blue, 25% 2-Mercaptoethanol (BME), H_2O. pH 6.8	Add BME to aliquot prior to use	
Non-permeabilization IF Buffer	1X PBS, 3% BSA		

Table 3. Plasmids

Construct	Vector	Gene	Origin
HA-GLUT4-GFP	pQBI25	GLUT4	Dawson Lab, (Dawson et al. 2001)
GIV-3XFLAG-wild type	pcDNA3.1-CMV	CCDC88a	Farquhar Lab, (Garcia-Marcos, Ghosh, and Farquhar 2009)
GIV-F1685A-3xFLAG	pcDNA3.1-CMV	CCDC88a mutant	Farquhar Lab, (Garcia-Marcos, Ghosh, and Farquhar 2009)
GIV-S1689D-3xFLAG	pcDNA3.1-CMV	CCDC88a mutant	Made in our lab, (Lopez-Sanchez et al. 2013)

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