Rac GTPase regulation of GLUT4 traffic in muscle cells:
mechanisms and implications

Ting (Tim) Chiu

A thesis submitted with requirements for the degree of Doctor of Philosophy in
Biochemistry
Graduate Department of Biochemistry
University of Toronto
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Doctor of Philosophy in Biochemistry, Graduate Department of Biochemistry,
University of Toronto
Ting (Tim) Chiu (2014)

Abstract

One of the hallmarks of postprandial glucose homeostasis is the ability of insulin to promote glucose uptake into skeletal muscles. Insulin achieves this feat by enhancing the recruitment of glucose transporter 4 (GLUT4) from an intracellular compartment to the plasma membrane of muscles in order to create a net increase in surface GLUT4, which results in elevated glucose uptake. From a molecular perspective, this insulin-regulated GLUT4 traffic action requires the independent activation of Akt and Rac-1 in muscle cells because perturbation of either molecule results in an impaired response. Although Rac-1 has been validated as key component of insulin response, its downstream signalling capacity contributing to GLUT4 translocation remains unexplored.

Studies on Rac-1 have shown that it is responsible for the formation of cortical remodelled actin that facilitates GLUT4 translocation following insulin stimulation. However, the downstream Rac-dependent molecules governing this actin remodelling are undetermined. Here we identified Arp2/3 and cofilin as the Rac-dependent regulators of insulin-induced actin remodelling in muscle cells. While Arp2/3 acts to initiate a burst of actin polymerization, cofilin balances out the actin dynamics through its severing/depolymerizing activity. Inhibition of either molecule’s function leads to defective GLUT4 translocation mediated by insulin in muscle cells, suggesting the requirement of actin dynamics to facilitate GLUT4 traffic to the plasma membrane.
Furthermore, given the importance of Rac-1 in insulin-mediate GLUT4 traffic, its application potential to reverse insulin resistance has never been explored. We discovered that providing muscle cells with additional Rac-1 activity produces an insulin-independent gain in surface GLUT4 with magnitude comparable to that normally elicited by insulin. This phenotype is accomplished because of the concomitant cross-activation of Akt pathway when supplying the cells with active Rac-1. Interestingly, this response can bypass signalling defects imposed by cellular insulin resistance conditions, leading to restoration of GLUT4 translocation in muscle cells.

Overall, these results not only reinforce the functional impact of Rac-1 on GLUT4 traffic but also identify additional molecules governed by Rac-1 contributing to the integrity of this insulin-mediated response in muscle cells.
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Preface

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Published papers


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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Akt1/2</td>
<td>Akt inhibitor 1/2</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>AS160</td>
<td>Akt substrate of 160kDa</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAR</td>
<td>Bin–Amphiphysin–Rvs</td>
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<tr>
<td>CA</td>
<td>Constitutively active</td>
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<td>CC</td>
<td>Compound C</td>
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<td>Carbohydrate responsive element binding protein</td>
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<td>Cdc42/Rac interacting binding domain</td>
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<td>Diaphanous autoregulatory domain</td>
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<td>Dbl homology</td>
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<tr>
<td>DID</td>
<td>Diaphanous inhibitory domain</td>
</tr>
<tr>
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<td>Dominant negative</td>
</tr>
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<td>DRF</td>
<td>Diaphanous-related formins</td>
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<td>FKBP</td>
<td>FK506-binding protein</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>GLUT4</td>
<td>Glucose transporter 4</td>
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<td>Guanosine triphosphatases</td>
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<td>Ras GTPase-activating-like protein</td>
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<td>IRAP</td>
<td>Insulin responsive aminopeptidase</td>
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<td>JMY</td>
<td>Junction-mediating regulatory protein</td>
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<td>NPF</td>
<td>Nucleation promoting factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>N-WASP</td>
<td>Neural WASP</td>
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<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
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<tr>
<td>PAS</td>
<td>anti-phospho Akt substrate antibody</td>
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<td>PMA</td>
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<td>Soluble NSF attachment protein receptor</td>
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<td>SRA1</td>
<td>Specifically Rac-associated protein 1</td>
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<td>Slingshot</td>
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<td>Transferrin</td>
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<tr>
<td>TGN</td>
<td>Tran-Golgi network</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection fluorescence</td>
</tr>
<tr>
<td>TOCA</td>
<td>Transducer of Cdc42-dependent actin assembly</td>
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<td>VAMP2</td>
<td>Vesicle-associated membrane protein 2</td>
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<td>WASP and SCAR homologue</td>
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<td>WASP homologue associated with actin, membranes and microtubules</td>
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<td>YF-CA-Rac lacking the CAAX motif</td>
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<tr>
<td>YF-Tiam1</td>
<td>YF-linked DH-PH domain of Tiam1</td>
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CHAPTER 1: Introduction
With increasing number of proteins being identified and their interactomes being characterized, the number of signalling cascade combination within a cell appears to be endless and extremely complex. Yet, the cell somehow knows precisely where, when, and how long to turn on the molecules in order to achieve the correct biological response. As the result, there must be delicate ways for the cell to regulate signals spatially-temporally. For this reason, cells are equipped with many proteins that function as molecular switches at distinct regions within the cell to allow timely signal transduction. One of such regulatory mechanisms is the spatial and temporal control of Rho guanosine triphosphatases (GTPases). Known as the masters of actin regulation, different members of Rho GTPase family must be activated at the right place and right time for the actin-dependent cellular processes to work. One of the most important biological responses that Rho GTPases and actin contribute to is the insulin-dependent recruitment of glucose transporter-4 (GLUT4) to the cell surface of muscle cells. This thesis will take a closer look at the interplay between Rho GTPases and actin during insulin stimulation and the role of the Rho GTPase-mediated changes in actin in the overall journey of GLUT4 vesicles to the plasma membrane.

1.1: Background on Rho GTPase family

Rho GTPases belong to a subclass of Ras superfamily of small GTPases, which consists of 5 major subclasses based on their sequence and function similarities: Ras, Rho, Rab, Arf, and Ran (1). They share conserved G box GDP/GTP-binding domains and GTPase motifs that allow them to function as molecular switches upon cycling of their GDP/GTP-bound forms. There are a spectrum of cellular functions controlled by
the Ras superfamily small GTPases. Rho GTPases in particular have received enormous research interests due to their unique control of the actin cytoskeleton.

To date, there are 22 mammalian genes encoding for Rho GTPases. Based on sequence similarities, they can be further categorized into 8 subgroups: Cdc42 [Cdc42, TC10, TCL (TC10-like), Chp, Wrch-1], Rac (Rac1–3, RhoG), Rho (RhoA–C), Rnd (Rnd1, Rnd2, Rnd3/RhoE), RhoD (RhoD and Rif), RhoH/TTF, Rho BTB (RhoBTB1 and RhoBTB 2) and mitochondrial Rho (Miro-1 and Miro-2) (2). Among the subgroups, Rho, Cdc42, and Rac are studied extensively due to the knowledge of their specific modulation of the actin cytoskeleton. Although other members have also been implicated in alterations in actin cytoskeleton, their precise biological functions are less understood.

1.2: Regulation of GTP/GDP cycles in Rho GTPases

Similar to other family of small GTPases, Rho GTPases act as molecular switches by cycling between the inactive GDP bound state and the active GTP bound state. However, due to the low intrinsic exchange and GTP hydrolysis rate, the guanine nucleotide cycle is regulated by additional proteins and covalent modifications (Figure 1-1).
Figure 1-1. The regulation of Rho GTPases. Rho GTPases switch between the inactive GDP- and active GTP-bound form with the help of GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) respectively. Prenylation of Rho GTPases and the interaction with guanine nucleotide dissociation inhibitor (GDI) regulate the membrane-association and cytoplasmic sequestration of Rho GTPases. Additional covalent modifications such as ubiquitination, phosphorylation, sumoylation, and palmitoylation impact the stability and the activation status of Rho GTPases.

1.2-1: GEFs and GAPs

Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP to GTP so that the Rho GTPases become active. Since the initial discovery of the Dbl protein that catalyzes the GTP loading of Cdc42 in *Saccharomyces cerevisiae* (3), the Dbl homology (DH) and adjacent C-terminal pleckstrin homology (PH) domain have been used as the conserved sequence marker to search for potential Rho GEFs. Up-to-date, 69 members of DH-PH containing GEFs have been identified with varying substrate specificities. More recently, Dock180 related proteins containing Dock
homology region-2 domains have emerged as a second family of GEFs for Rho GTPases (4). Nonetheless, both families initiate GTP loading by promoting an intermediate nucleotide-free state in the GTPases during which GTP binds to the GTPases due to its higher concentration than that of GDP in cells.

On the contrary, GTPase activating proteins (GAPs) inactivate Rho GTPases by stimulating their intrinsic GTPase activity to promote the hydrolysis of GTP to the inactive GDP. Despite having identified ~70 GAPs containing RhoGAP domains, the precise biological function of each member remains largely elusive due to the functional redundancy that exists within this large family.

1.2-2: Prenylation

In addition to controlling the magnitude of Rho GTPase activation via the guanine nucleotide exchange, the specific subcellular localization of the GTPases is also a major determinant of their biological function within a cell. At a given time, only a small portion of the total Rho GTPases is active and anchored to the plasma membrane or endomembrane. The membrane localization cue is controlled by the post-translational lipid modification on the C-terminal CAAX motif of the Rho GTPases (C = cysteine, A = aliphatic amino acid, and X = terminal amino acid). Depending on the amino acid at X, the cysteine residue is covalently modified with either a farnesyl or geranylgeranyl isoprenoid lipid to enhance the hydrophobicity of the GTPase and its membrane insertion (5).

1.2-3: GDI

In contrast to lipid modification, Rho specific guanine nucleotide dissociation inhibitors (GDIs) work in reverse to sequester the majority of the Rho GTPases inactive
in the cytoplasm. The interaction between Rho GTPases and RhoGDIs not only prevents the nucleotide exchange but also inhibits the GTPase activity. Having a higher affinity for GDP bound Rho GTPases in a membrane environment, RhoGDIs are capable of extracting inactive Rho GTPases from the membrane by shielding the isoprenyl moiety away from water to maintain a stable folding of Rho GTPase in the cytoplasm. This function enables GDI to shuttle Rho GTPases between cytosol and the membrane compartments as inactive and active pools, respectively.

1.2-4: Covalent modifications and miRNAs

Additional regulation arises from covalent modifications of Rho GTPases and the control of their expression. Phosphorylation of Rho GTPases by different kinases such as cAMP dependent kinase PKA, cGMP dependent kinase PKG, Src tyrosine kinase, and Akt have been reported to increase interactions with RhoGDI and reduce GTP binding capacity (6-8). Ubiquitination involves the covalent attachment of the 8 kDa ubiquitin polypeptide to lysine residues on the target molecule to trigger degradation controlled by ubiquitin–proteasome system. Ubiquitination of Rho GTPases was suggested by the reduction or enhancement in the amount of Rho GTPases upon changes in the expression of ubiquitin ligase (9). Sumoylation and palmitoylation of Rac1 have also been reported and they assist in optimal loading of GTP and stability of Rac1 at the membrane, respectively (10,11). To control the protein expression level, endogenous microRNAs (miRNAs) silence the targeted genes by degrading their mRNAs to suppress protein translation. Changes in Rho GTPases miRNA levels leading to fluctuating amount of Rho GTPase expression can be observed in disease development and cell proliferation (12).
1.3: Cellular functions of Rho GTPases

Years of research have discovered the participation of Rho GTPases in numerous cellular processes such as cell-cycle progression, cellular morphogenesis, gene transcription, cell adhesion and migration, vesicle traffic, and enzymatic activities. The contribution of Rho, Cdc42, and Rac towards each category is summarized in Table 1.

Table 1. Summary of Rho GTPases-regulated cellular processes.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Functions</th>
<th>Rho GTPases and Mechanisms</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>G1 progression</td>
<td>• Rac/Cdc42: control cyclin D expression, down-regulate CDK inhibitor expression</td>
<td>(13,14)</td>
</tr>
<tr>
<td></td>
<td>Mitosis</td>
<td>• Cdc42: mediate microtubule connection to kinectochore via mDia3</td>
<td>(15)</td>
</tr>
<tr>
<td></td>
<td>Cytokinesis</td>
<td>• Rho: govern actin-myosin contractile ring at the cleavage furrow</td>
<td>(16)</td>
</tr>
<tr>
<td>Cell morphogenesis</td>
<td>Cell-cell adhesion</td>
<td>• Rho/Cdc42/Rac: assist in formation of adherent junction through actin cytoskeleton</td>
<td>(17)</td>
</tr>
<tr>
<td></td>
<td>Cell polarity</td>
<td>• Rho/Cdc42/Rac: regulate the formation of tight junction via actin cytoskeleton</td>
<td>(18)</td>
</tr>
<tr>
<td>Cell movement</td>
<td>Cell migration</td>
<td>• Rho/Rac: stimulate retraction and protrusion at the rear and front of the cell respectively</td>
<td>(19)</td>
</tr>
<tr>
<td></td>
<td>Directionality</td>
<td>• Cdc42: maintain cell polarity in the direction of chemoattractant-gradient</td>
<td>(20)</td>
</tr>
<tr>
<td>Vesicle traffic</td>
<td>Golgi-ER transport</td>
<td>• Cdc42: interact with COPI to promote actin and microtubule dependent vesicle transport</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>Exocytosis</td>
<td>• Rho/Rac: modify actin cytoskeleton for vesicle traffic</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>Endocytosis</td>
<td>• Rho/Cdc42/Rac: modulate endocytic processes through actin reorganization</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>Enzymatic activity</td>
<td>NADPH oxidase</td>
<td>(22)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Rac: increase ROS production</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PI5K</td>
<td>(23)</td>
</tr>
</tbody>
</table>
1.4: The dynamic control of actin cytoskeleton

Despite the complexity of biological functions in which Rho GTPases participate, the majority of the changes arise because of their impact on actin cytoskeleton. Actin is one of the most abundant and evolutionarily conserved proteins in the cell. It is a 42kDa ATP-binding protein that exists as monomeric globular actin (G-actin) and assembled into filamentous actin (F-actin). Due to the intrinsic structural polarity of G-actin and the head-to-tail polymerizing orientation, the assembled F-actin is also polarized, with a growing barbed end (+ end) and a trailing pointed end (- end). While ATP bound G-actin is incorporated to extend F-actin at the barbed end, previously assembled G-actin at the pointed end undergoes ATP hydrolysis to create an ADP-bound state. This hydrolytic property creates an ATP-actin- and ADP-actin-rich zone at the barbed and pointed ends, respectively. Under this polarized biochemical and structural constraint, actin polymerization becomes favourable at the ATP barbed end while actin depolymerization dominates at the ADP pointed end (Figure 1-2).

In the cell, the dynamic actin turnover is the main driver of the actin cytoskeleton network which remodels to create structural scaffolds, mechanical force, and tracks for traffic events. These fundamental properties contribute significantly to biological functions such as morphogenesis, migration, cytokinesis and vesicle transport. Needless to say, emphasis has been placed on identifying various actin-binding molecules that regulate this dynamic behaviour and their mechanisms of action. In particular, studies on proteins that control actin polymerization or depolymerization have provided insights on this tightly regulated intricacy of F-actin network (Figure 1-2).
Figure 1-2. Regulation of actin dynamics. F-actin is assembled from monomeric G-actin to create a polymerizing barbed end rich in ATP-actin and a depolymerizing pointed end with ADP-actin. The growth of F-actin can be accomplished through either spontaneous, branched actin, or linear actin polymerization. This increase in actin assembly is balanced out by the severing/depolymerisation at the pointed end to regenerate a free pool of G-actin ready for additional rounds of actin growth. F-actin bundling proteins can crosslink assembled F-actin to provide strengthened structural integrity. Proper control of these regulatory steps determines the overall dynamics of actin in the cell.

1.4-1: Actin polymerization

Spontaneous actin polymerization involves two main stages, nucleation and elongation, before finally reaching to a steady state level in which no net filament growth is obtained. Nucleation begins with the formation of G-actin dimer/trimer. However, this step is often rate limiting due to the instability of the trimer complex (24). Therefore,
factors such as monomeric actin binding molecules, which control the availability of G-actin, and actin nucleators, which stabilize the initial actin multimer, would influence the rate of actin polymerization.

1.4-1-I: Monomer actin binding proteins: thymosin-β4 and profilin

Thymosin-β4 and profilin interact with monomeric actin by competing for an overlapping binding site on G-actin. The exchange rate of G-actin between the two molecules is rapid due to the high dissociation constants of actin with either protein (25). The interaction of thymosin-β4 and profilin with G-actin makes them the two major proteins responsible for maintaining a pool of monomeric actin in the cytosol and limiting filament spontaneous polymerization. On their own, thymosin-β4 and profilin have inhibitory and promoting actions towards actin polymerization, respectively. While thymosin-β4 maintains G-actin in ADP-bound form, profilin catalyzes the exchange of ADP to ATP on the associated actin monomer (26,27). At the same time, profilin prevents the intrinsic hydrolytic activity of actin so that the G-actin is primed with ATP for additional rounds of actin polymerization at the barbed ends.

1.4-1-II: Actin nucleators
1.4-1-II-A: Formin

In mammalian cells, there are 15 different formins which can be divided into 2 large subfamilies, formins and Diaphanous-related formins (DRFs) (Figure 1-3). They share the formin homology (FH) domains FH1 and FH2 but differ in their N-terminal regulatory domains. In all formins, the FH2 domain and its ability to homodimerize are essential for formin-mediated actin assembly (28,29). Structurally, the FH2 domain interacts directly with the actin polymerization dimer intermediate and stabilizes it to
accelerate polymerization (30). The FH1 domain, on the other hand, associates with profilin via its proline-rich segment and brings ATP-actin in close proximity to the actin assembly site. Given their higher affinity for ATP-bound actin at the barbed end of F-actin, formins move along with the growing filament to allow rapid addition of G-actin. This processive activity also prevents capping proteins from binding to the barbed end and stopping filament growth, which enables the formation of longer F-actin structures like stress fibers and filopodia (31). It is because of this elongation property that formin has been implicated in cellular functions involving actin cables, filopodia, stress fibers, actin-rich cell adhesions, and cytokinesis.

DRFs are better understood than formins due to their characterized regulatory cues. In resting cells, the C-terminal diaphanous autoregulatory domain (DAD) binds with the N-terminal diaphanous inhibitory domain (DID) to create an autoinhibitory state (32). Only upon binding of active Rho GTPases to the adjacent GTPase-binding domain (GBD) would the molecule become activated. Depending on the members of DRFs, interaction with different Rho GTPases (RhoA-C, Cdc42) to the GBD can alleviate the autoinhibitory conformation (33). However, additional factors are required to achieve a full activation because only partial actin polymerizing activity was obtained with the addition of Rho GTPase to DRFs in vitro (34).
Figure 1-3: Domain structures of formins and DRFs. DAD and DID interaction confers DRFs in an autoinhibitory state at rest. Binding of GTP-loaded Rho to GBD releases this inhibitory association leading to activation. FH: formin homology domain, GBD: GTPase binding domain, DID: diaphanous inhibitory domain, DAD: diaphanous autoregulatory domain.

1.4-1-II-B: Arp2/3

Arp2/3 is a 220kDa complex consisting of 7 subunits (ARP2, ARP3, ARPC1-5) that are evolutionarily conserved among the eukaryotic cells (35). It functions by binding to the existing F-actin at the pointed end to initiate new branched filament formation at a 70° angle. Structurally, ARP2 and ARP3 are actin-related proteins that also harbor ATP-binding ability. They orient themselves to form the initial dimer of the new branched filament while ARPC1-5 positions the complex stably on the existing filament. By itself, Arp2/3 is relatively inefficient at promoting nucleation and the subsequent actin polymerization. Although phosphorylation of ARP2 has been reported to increase its binding to the filament (36), the most well characterized mechanism to increase its activity is via the engagement with nucleation promoting factors (NPFs).

Most NPFs contain a WCA domain that is comprised of WASP homology 2 (WH2), amphipathic connector region, and acidic peptides. The CA portion interacts with multiple subunits of Arp2/3 to exert conformational changes that prime ARP2 and
ARP3 for nucleation while the W region binds and delivers G-actin to the barbed end of ARP2-ARP3 dimer.

There are two classes of NPFs. The class I NPFs display a C-terminal WCA domain while diversified N-terminal regions confer to them differential regulations. Members of class I NPFs include: WASP and neural WASP (N-WASP); WASP-family verprolin homologue (WAVE/SCAR), WASP and SCAR homologue (WASH), WASP homologue associated with actin, membranes and microtubules (WHAMM), and junction-mediating regulatory protein (JMY). The class II NPF, cortactin, contains only the acidic peptide portion of WCA domain but retains the ability to interact with Arp2/3. The overview of each NPF is illustrated in Figure 1-4 and each member will be described in the following section.
The common feature among all the member of NPFs is the ability to interact and stabilize Arp2/3 via the WCA domain or the A domain, in the case of cortactin, for the initiation of branched actin formation. The signature behind each NPF’s activity, stability, and cellular localization is determined by the variable functional domains situated N-terminal to the WCA region. PRD: proline-rich domain, CRIB: Cdc42/Rac-interacting binding domain, B: polybasic region, I: autoinhibitory motif, WH2: Wasp homology 2, SHD: SCAR homology domain, WHD: WASHhomology domain, TBR: tubulin binding region, CC: coiled-coiled domain.

1.4-1-II-B.1: WASP and N-WASP

WASP is specifically expressed in haematopoietic cells while N-WASP is found in most cell types. Both of them contain the N-terminal WASP homology 1 domain, a basic region, a Cdc42/Rac-interacting binding domain (CRIB), an autoinhibitory motif, and a proline-rich domain (PRD) followed by the characteristic WCA region. At rest, WASP remains inactive due to the intramolecular interaction between the WCA and its autoinhibitory region. This inactive state is further stabilized by WASP-interacting protein (WIP) binding to the WH1 domain (37). Upon growth factor stimulation, binding
of active Cdc42 to the CRIB domain causes conformational changes that relieve the steric hindrance on WCA (38). Additional regulatory components, such as \( P_{i(4,5)}P_2 \) interaction with the basic residues of WASP, SH3-containing proteins binding to PRD, and inducible clustering of WASP molecules at the membrane, integrate together with Cdc42-dependent control to promote higher activity from WASP family proteins (39-41). WASP and N-WASP participate in a vast number of actin-dependent cellular processes. Disruption of WASP/N-WASP results in impaired filopodia formation, dorsal membrane ruffling, membrane invagination, and endocytosis (42).

1.4-1-II-B.2: WAVE

Three isoforms of WAVE are expressed in mammalian cells. WAVE1 and WAVE2 are distributed in most cell types but all three isoforms appear to concentrate in the brain. Unlike WASP, WAVE lacks the CRIB domain to interact with Rho GTPases but contains an N-terminal \( \text{SCAR homology} \) domain (SHD), a basic region, and a PRD precedes the common terminal WCA domain. Through this unique SHD, WAVE forms a heteropentameric complex by associating with BRICK1, ABI1, \( \text{NCK-associated protein} \) 1 (NAP1) and specifically \( \text{Rac-associated protein} \) 1 (SRA1). This multimeric state is crucial for the stability of WAVE because perturbation of any subunit disrupts the function and localization of the whole complex (43,44). Similar to WASP, WAVE is natively inactive due to masking of WCA domain by SRA1/NAP1 subunits (45). Several factors assist the activation of WAVE. GTP-bound Rac binding to SRA1, \( P_{i(3,4,5)}P_3 \) interaction with the basic residues, IRSp53’s association with PRD, and phosphorylation act synergistically to induce conformational changes in WAVE that favour the interaction with Arp2/3 to enact actin branch polymerization (46-48). The main function of WAVE is
to generate lamellipodia and plasma membrane protrusions for cell migration. Although there are functional similarities among the WAVE isoforms, the redundancy is not sufficient to prevent defects that manifest in individual isoform gene knockout conditions.

1.4-1-II-B.3: WASH

Initially discovered as one of several subtelomeric genes, WASH was found to have actin polymerization capacity driven through Arp2/3 (49). In addition to the conserved WCA element, it also encodes an N-terminal WASH homology domain 1, and a tubulin binding region followed by a PRD. Similar to WAVE, WASH is found in a multiprotein complex that consists of FAM21, Strumpellin, SWIP, Ccdc53, and capping protein (CapZ). Although the precise function of each subunit associated with WASH is not fully understood, it is appreciated that they contribute to the overall stability of the complex, as down-regulation of one affects the expression of others (50). Depending on the purification techniques, it is debated whether the WASH complex is natively active or inhibited like the WAVE multimers. The precise mode of WASH activation is not clear. Nonetheless, the association of capping protein CapZ with WASH appears to enhance the Arp2/3-dependent actin polymerization (51). Functionally, WASH is uniquely positioned in early and slow recycling endosomes via the interactions between retromer complex subunit VPS35 and its FAM21 subunit and the association with microtubules through its tubulin binding region (52-54). This specific localization primes WASH to exert actin-dependent control over endosomal shape, biogenesis, and traffic.

1.4-1-II-B.4: WHAMM
WHAMM is another protein discovered during bioinformatics searches for proteins containing WCA domain. It is ubiquitously expressed and contains N-terminal WHAMM membrane interacting domain and a coiled-coiled domain preceding the WCA region. The regulatory mechanism for WHAMM’s activity *in vivo* is not fully understood but it is not autoinhibited like the WASP family nor does it exist in a complex like WAVE and WASH. When expressed exogenously in cells, it localizes to the cis-Golgi and tubulovesicular ER-Golgi intermediate compartment (55). Such distribution pattern is the result of binding to microtubules via its coiled-coiled domain and the interaction between the WHAMM membrane-interacting domain and the Golgi tethering factor. The unique placement of WHAMM allows it to control Golgi morphology and ER-Golgi transport in an actin- and microtubule-dependent manner (55).

1.4-1-II-B.5: JMY

Although JMY has the conserved WCA domain, it also encodes two additional WH2 domains separated by a linker region directly N-terminal to WCA to create a WWLWCA segment. This special arrangement enables JMY to initiate actin nucleation by both Arp2/3-dependent and Arp2/3-independent mechanisms (56). While the Arp2/3-mediated polymerization is derived from the WCA of JMY, the Arp2/3-independent mode arises from the ability to cluster 3 actin monomers closer to one another due to the proximity of the 3 WH2 domains. This is similar to the mechanism of other Arp2/3-independent nucleation molecules, such as formin and Spire, relying on means of three or four clustered actin monomers (57). Nonetheless, the functional relevance of JMY is still under investigation. Its ability to act also as a co-factor for the transcriptional
regulator of p53 has suggested the potential of integrating actin cytoskeletal changes with stress responses (58).

1.4-1-II-B.6: Cortactin

Cortactin differs from the class I NPFs by not encoding the full WCA domain. Instead, it contains only the acidic portion at the N-terminus, which retains the ability to interact with Arp2/3, a middle repeating sequence responsible for F-actin binding, a PRD domain rich in phospho-regulatory sites, and an SH3 domain at the C-terminus. By itself, both the acidic region and F-actin binding repeats are required for the activity of cortactin towards Arp2/3 but it has a weaker nucleation promoting action compared to N-WASP (59). Nonetheless, interactions between the cortactin SH3 domain and PRD of N-WASP can liberate N-WASP from its autoinhibitory state. This complex formation between cortactin and N-WASP allows synergistical enhancement of Arp2/3’s nucleation activity (60). Complicated phospho-regulatory mechanisms with both positive and negative effects have been proposed based on identified serine and tyrosine residues on cortactin (61). Their precise contribution will most likely be context- and stimulus-specific. Functionally, cortactin is involved in invadopodia formation (62). Although it has also been implicated in membrane protrusion, the main contribution of cortactin is towards lamellipodia persistency rather than formation by promoting new adhesion at actin-based protrusion sites (63).

1.4-1-II-C: WH2-repeat nucleators

More recently, a new class of nucleators was identified that is characterized by a common repeating cluster of 3 or more G-actin binding motifs, such as WH2. These
proteins exert nucleation activity by recruiting multiple actin monomers in close
proximity and organizing them into a nucleation complex (64). Members of this family
include spire, cordon-bleu, leiomodin, and interestingly some bacterial nucleators (42).
Little is known about their regulatory mechanism and exact biological functions except
for cordon-bleu’s involvement in neurite branching and leiomodin’s role in sacromeric
actin organization due to their respective high expression in hippocampal neurons and
skeletal/cardiac muscles (65,66).

1.4-2: Severing and depolymerization

Actin turnover is a dynamic process in which the polymerization needs to be
properly balanced by breaking down F-actin via severing and depolymerization in order
to regenerate the free pool of G-actin for future assembly. Because growth at the
barbed end is relatively fast and can be accelerated by the nucleators, other factors are
also required to increase the rate of depolymerization at the pointed end to maintain the
equilibrium. Besides liberating free G-actin, severing can also promote subsequent
polymerization by increasing the number of plus ends where actin monomers can be
newly incorporated. Two major molecules responsible for severing and
depolymerization include gelsolin and actin depolymerizing factor (ADF)/cofilin.

1.4-2-I: Gelsolin

Gelsolin is characterized by the 6 gelsolin-like domain (G) that mediates calcium-
dependent severing and capping. Biochemical analysis reveals the presence of three
actin binding sites including G1: calcium-independent binding to G-actin, G2-3: calcium-
independent binding to F-actin, and G4-6: calcium-dependent binding to G-actin (67).
G1-3 is the severing component while G4-6 acts in cooperative manner to assist the
activity (68). Intramolecular interaction between the C-terminal tail and G2 normally keeps gelsolin in an autoinhibitory form. Upon binding of calcium, a conformational change relieves this constrain and increases gelsolin's affinity for the ADP-associated actin filament to initiate active severing and capping (69-71). However, in the presence of PI_{4,5}P_{2}, its severing activity is reduced due to decreased binding to F-actin (72).

1.4-2-II: ADF/Cofilin

ADF/cofilin is an actin-binding family protein that possess an ADF-homology domain. Members of this family function to sever aged filaments and induce depolymerization to replenish free monomeric actin. It binds to both F-actin and G-actin via the G/F binding site, which is critical for its severing and depolymerizing activity (67). The major biochemical difference between ADF and coflin is that ADF causes a greater steady-state actin depolymerization than coflin (73). The critical regulatory component for ADF and coflin is centred on their conserved Ser-3 located at the N-terminal G/F site. Phosphorylation of Ser-3 significantly reduces the ability of ADF and coflin to bind to actin leading to the reduced activity while dephosphorylation promotes their activation (74). While kinases like LIMK and testicular protein kinase are responsible for the phosphorylation of ADF/cofilin (75,76), phosphatases such as slingshot and chronophin act to counter their effect (77,78). Interestingly, LIMK is a downstream effector of Rho-associated kinase (ROCK) and p21-activated kinase (PAK), which are controlled by Rho and Cdc42/Rac respectively (79,80). This allows for a Rho GTPase-dependent influence on ADF/cofilin activity. Other regulatory mechanisms include decrease in actin binding and severing upon association with PI_{4,5}P_{2} and stronger depolymerizing activity at basic pH (81,82). However, the pH effect is more limited to ADF compared to coflin.
In addition, competitive actin-binding can also influence the activity of ADF/cofilin. Tropomyosin competes with ADF/cofilin on F-actin binding to stabilize the filament while profilin extract depolymerized G-actin from ADF/cofilin to induce nucleotide exchange to generate an ATP-bound actin for subsequent polymerization (83,84). Although additional molecules such as actin interacting protein 1 and coronin act to augment ADF/cofilin’s filament turnover activity, the exact mechanism behind this stimulatory effect remains to be defined (85).

1.4-3: Other actin modulations - capping protein (CP) and bundling protein

As the name suggests, the heterodimer CP functions to cap barbed ends to inhibit filament growth. At the same time, it prevents depolymerization occurring at the capped end, leading to preferred breakdown at the pointed end (86). Interaction with PI(4,5)P_2 has been demonstrated to inhibit the capping ability and is hypothesized to be a key element in generating membrane protrusion where active polymerization is required (86). Additional regulatory molecules, such as CARMIL and CKIP-1, can associate with CP to interfere with its binding to the barbed end (87,88).

F-actin can be further packaged into superstructures by the help of actin bundling proteins. They act to form tight parallel actin bundles or looser orthogonal meshworks via their two actin binding sites to crosslink actin filaments. Such structures provide improved cellular rigidity and mechano-sensing ability that ultimately contribute to biological functions such as invadopodia/filapodia formation, cell-cell junction integrity, stress fiber and cortex maintenance. Various proteins including, spectrin, filamin, alpha-actinin, dystrophin, myosin II, and fascin, have been identified to have this bundling ability and their respective function depends on the cellular context (89).
In order for actin to achieve its diversified biological functions that require dynamic filament turnover at specific locations within a cell, molecules that modulate actin dynamics need to be precisely governed in a spatial-temporal manner. In accordance with these criteria, Rho GTPases are often referred to as the master regulators of actin because of their ability to function as switches that can be acutely turned on and off in a timely controlled fashion. Many of the above mentioned molecules that regulate actin dynamics are under the stringent control of Rho GTPases (Figure 1-5).

Active RhoA can turn on ROCK by relieving it from its autoinhibitory state. ROCK enhances myosin activity by direct phosphorylation of MLC and inhibition of the MLC phosphatase function via phosphorylation of its myosin phosphatase-targeting subunit 1 (90,91). This strengthens the actin-myosin interaction for force generation. In addition, ROCK can induce the activation of LIMK to prevent the severing activity of cofilin via LIMK-regulated phosphorylation (92). Furthermore, mDia1 is activated upon binding of active RhoA to stimulate longitudinal F-actin polymerization (93). All together, these controls by RhoA initiate stress fiber formation/stability, cytokinesis, and cell polarity during migration by facilitating detachment at the trailing end.

Cdc42 governs actin dynamics by interacting with nucleation promoting factors N-WASP and mDia to stimulate branched and linear F-actin formation, respectively. Moreover, Cdc42 binds to PAK to mediate LIMK activation and its subsequent phospho-regulation on cofilin’s severing activity. These Cdc42-mediated actin responses
contribute to filapodia formation, neurite extension, and directional sensing during migration (94).

Rac initiates Arp2/3 dependent actin branching and polymerization, specifically by engaging in WAVE to promote the augmented activity. Similar to Cdc42, Rac can activate PAK and can limit cofillin activity via LIMK. Rac’s activity towards actin is crucial for lamellipodia formation, membrane protrusion, axon growth and migration (94).

**Figure 1-5: Rho-dependent signalling regulation on actin dynamics.** Rho, Cdc42, and Rac engage in different downstream molecules such as ROCK, mDia, WASP, WAVE, or PAK to initiate the chain of actin modifying events that contribute to actin dynamics in a cell at a given moment.

1.5-1: Vesicle traffic regulated by Rho GTPase-mediated actin dynamics

All together, the precision of controls on actin filament dynamics establishes the basis of actin-regulated cellular biology. Of the actin-dependent functions, a fundamental aspect is the regulation on vesicle traffic. Eukaryotic cells are composed of distinct membrane-enclosed compartments/organelles that actively communicate with
one another through the exchange of protein-containing vesicles. These carrier vesicles relay information and content among the different organelles through endosomal traffic and communicate with the extracellular milieu via vesicle-mediated exocytosis and endocytosis at the plasma membrane. Although Rab family proteins are considered as the guiding cues for vesicle sorting, Rho GTPase-dependent F-actin reorganization has also been implicated in assisting a diversity of vesicle traffic steps.

1.5-1-I: Exocytosis

During exocytosis in neuroendocrine cells, intrinsic cortical actin acts as a barrier that must be broken down via activation of severing molecules such as scinderin, a gelsolin-family protein, in order to release vesicles for fusion with the plasma membrane (95). However, actin polymerization also plays a role in exocytosis as evident by the requirement of Cdc42- and Rac-dependent Arp2/3 polymerization at the exocytic sites (96-98). For the release of larger granules, an actin coat has been visualized to form around the base of the fused vesicles to provide additional stability for fusion and to facilitate content expulsion via actin-myosin-dependent force generation (99-101). This de novo actin coat assembly is driven by the activity of Cdc42 and the subsequent recruitment of N-WASP and Arp2/3 (102). Accordingly, neutrophils from Rac-2 null mice exhibit reduced granule release, likely due to defective actin dynamics during the exocytic process (103). In addition to the contribution by branched cortical actin to vesicle fusion at the plasma membrane, linear F-actin polymerization induced by spire and formin can serve as tracks for long range transport of vesicle to the plasma membrane (104).

1.5-1-II: Endocytosis
In the process of vesicle internalization from the plasma membrane, force generated via actin polymerization pushes outwards to the membrane to propel the formation of the endocytic vesicle (105). This actin-mediated action for endocytosis is completely required in yeast but has varying degree of participation in mammalian cells depending on the amount of membrane tension the cell encounters (106). Nonetheless, some evidences support the participation of Cdc42-stimulated actin polymerization during clathrin-mediated endocytosis via interaction with intersectin, which is a scaffolding modulator in the formation of clathrin-coated vesicles (107). As endocytic processes involve bending of the membrane, BAR domain-containing proteins are positioned to stabilize the membrane curvature required for membrane tubulation prior to vesicle formation. One of such molecules, TOCA-1, contains an F-BAR domain for curvature sensing and also associates with Cdc42 to stage N-WASP->Arp2/3 F-actin generation to assist in membrane tubulation (108).

1.5-1-III: Endosomal sorting

Once internalized, vesicles need to be sorted through distinct endosomal compartments before arriving at their defined destinations within the cell. During this process, endosome-associated annexin A2 triggers spire and Arp2/3-mediated actin polymerization that is required for endosomal intermediate maturation and the eventual transport from early to late endosomes (109). Furthermore, active RhoB localized to early endosomes can delay the endosomal traffic of endocytosed vesicles by inducing association of vesicles with mDia1-generated F-actin (110).

The Trans-Golgi Network (TGN) often functions as a hub for vesicle sorting and its integrity and function is regulated by Arf. Interestingly, Arf can mediate the
recruitment of various RhoGEF and RhoGAP that affects the activity of local Cdc42 residing at the TGN via binding to γCOP, a COPI-vesicle coat protein. The dynamic regulation of Cdc42 activation status triggers Arp2/3-dependent actin polymerization that contributes to TGN morphology and vesicle sorting out of the TGN (111,112). Such actin-regulated Golgi function can also be affected by cofilin-mediated local actin dynamics as inhibition of cofilin reduces calcium uptake into the TGN via ATPase SPCA1, which in turn results in defective vesicle sorting (113).

All these findings point to the contribution of Rho GTPase-mediated actin dynamics in ensuring the proper traffic of vesicles at the cellular level. In a broader physiological context, regulated vesicle traffic event has profound effects on the outcome of biological functions including neurotransmitter release from synapses, hormone secretion into the blood stream, recruitment of immune cells for inflammatory responses and many more. Of interest here is the ability of insulin to actively recruit glucose transporter 4 (GLUT4)-containing vesicles from an intracellular compartment to the plasma membrane of muscle in order to enhance the rate of glucose clearance from the blood. The main focus of this thesis is to understand whether Rho GTPases and their actin-dependent function influence the insulin-stimulated traffic event of GLUT4-containing vesicles.

1.6: Regulation of glucose homeostasis by GLUTs

Glucose has evolved to be the major fuel source to maintain energetic integrity in higher organisms. Not only is glucose one of the key carbon sources for energy production, it and its metabolized intermediates can also serve as signalling molecules for gene expression, enzyme activity, and hormonal secretion. For example,
carbohydrate response element binding protein (ChREBP) exerts transcriptional control on glycolytic and lipogenic gene expression while glycolytic production of NAD+ regulates Sirt1 deacetylase activity (114,115). In addition, glucose-sensing pancreatic β-cells produce insulin in response to rising blood glucose. These functional impacts of glucose utilization impose a necessity for proper whole body glucose homeostatic regulation.

Intake of glucose into most tissues is governed by the availability of glucose transporters at the cellular surface. Since the discovery of GLUT1 as the 12-transmembrane facilitative transporter of glucose in the erythrocytes, thirteen other members of the GLUT/SLC2A family were identified in humans (116). Phylogenetically, they can be subdivided into class I (GLUT1, 2, 3, 4, 14), class II (GLUT5, 7, 9, 11) and class III (GLUT6, 8, 10, 12, 13) (117). GLUT1 is ubiquitously expressed with Km around 5mM (117). Most importantly, it mediates the rate-limiting step of supplying glucose fuel to proliferating cells during development, erythrocytes, cardiac muscles, blood-brain barrier endothelial cells, and astrocytes in central nervous system. The amount of cell surface GLUT1 is governed by its expression level within a given cell. Conditions such as hypoglycemia and hypoxia can induce an increased expression of GLUT1 in blood-brain barrier endothelial cells and cancer tumors respectively to achieve enhanced absorption of glucose (118,119).

Conversely, GLUT2 has an unusually high Km (~11mM) compared to any other GLUTs, which makes it the predominant transporter in tissues exposed to elevated glucose concentration such as intestine, pancreas, kidney, and liver. The high Km shifts the rate determining step in glucose metabolism to the phosphorylation of glucose by
glucokinase upon glucose entry to the cytosol, which traps it within the cell. Functionally, GLUT2 is crucial in glucose reabsorption by the kidney as lost-of-function mutations in GLUT2 severely impair this process (120). In intestines, GLUT2 is found at the basolateral membrane of enterocytes to transport glucose to the blood stream. However, GLUT2 can also be enriched at the apical surface upon detection of high postprandial sugar concentration in the intestinal lumen to boost glucose absorption together with the resident Na+/glucose co-transporters (121). In pancreatic β-cells, glucose metabolism increases the cytoplasmic ATP/ADP ratio, which induces membrane depolarization and the release of insulin-containing granules (122). Disruption of this glycolytic flux in GLUT2 knockout mice results in impaired insulin secretion (123). Nonetheless, this defect is more pronounced in rodents than in humans.

In addition to GLUT1, GLUT3 is another principal glucose transporter in neuronal cells and its high affinity for glucose (Km = ~1.4mM) among class I GLUTs is ideally suited to bring in glucose from the cerebrospinal fluid into neurons, as the ambient glucose concentration surrounding neurons is about 1-2mM (124). GLUT3 is also in expressed in platelets, lymphocytes and monocytes. In these cell types, GLUT3 is uniquely present in an intracellular compartment that undergoes regulated-translocation to the cell surface upon external stimulations such as PMA and LPS (124,125). Although the mechanism behind GLUT3-containing vesicle traffic is relatively unknown, the increased glucose uptake induced by GLUT3 provides the surge in energy demand to initiate clot formation by platelets and immune responses by monocytes.

GLUT4 (Km = ~5mM) is the major transporter in muscles and adipocytes, where it is responsible for the insulin-responsive glucose uptake. Among all the GLUTs,
GLUT4 is the most studied because of its importance in whole body glucose homeostasis, its intricate signal regulation on GLUT4-containing vesicle traffic by insulin, and its implication in insulin resistance and type 2 diabetes. Abrogation of GLUT4 function results in hyperglycemia and associated diabetic complications.

Not all GLUTs transport glucose as their preferred substrate. GLUT5, for example, has higher affinity for fructose and is responsible for dietary absorption at the apical membrane of intestinal epithelial cells. GLUT9, on the other hand, transports urate in liver and kidney to maintain uric acid level in the blood. Other GLUTs (6-8, 10-14) are less characterized and their physiological contributions remain to be determined.

The regulation of each GLUT directly influences the rate at which glucose is taken up into the respective tissues. For the majority of the GLUT family members, the determining regulation impinges on their level of expression which ultimately reflects the amount of GLUT proteins at the cell surface at a given time. GLUT4 stands out from other GLUTs in terms of the modes of its regulation. A key distinction is that the majority of GLUT4 is normally sequestered in intracellular pools and it is only recruited to the plasma membrane upon stimulation by insulin or other selective stimuli such as muscle contraction and energy deprivation. In combination with insulin’s major role in whole body glucose homeostasis, this unique traffic feature of GLUT4 has received enormous amount of research attention.

1.6-1: GLUT4 traffic

GLUT4 is a key component of postprandial insulin-mediated blood glucose homeostasis. The principal mechanism by which insulin enhances the uptake of dietary
glucose is by rapidly increasing the net amount of GLUT4 at the plasma membrane. This is achieved through the recruitment of GLUT4-containing vesicles from their intracellular compartment. Although this response takes place in both adipocytes and muscles, muscles account for the majority (>2/3) of insulin-induced glucose uptake in the body (126-128). This physiological trait of muscle makes GLUT4-dependent glucose regulation by insulin a key area of research interest.

To study GLUT4 traffic in muscle cells, our lab has pioneered the GLUT4myc cell line in which a myc-tagged GLUT4 construct is stably expressed in rat L6 myoblasts (129). This enables both population and single cell based assessment of the amount of GLUT4 at the plasma membrane via colourimetric and immunofluorescent assays. The gain in surface GLUT4 following insulin stimulation in the GLUT4myc cell line is comparable to that mediated in isolated skeletal muscles, which suggests a similar insulin-induced traffic between the two systems (130). As a result, the cell line represents a simpler model for molecular study on insulin actions in comparison to using the complicated skeletal muscles. This thesis focuses on the mechanism whereby GLUT4-containing vesicles are mobilized to the cell surface of muscle cells in response to insulin in L6 GLUT4myc muscle cell line.

1.6-1-I: Recycling GLUT4 vesicles versus GLUT4 storage vesicles

To understand the molecular underpinnings of the insulin-dependent recruitment of GLUT4 vesicles, studies have relied on cell cultures of adipocytes and muscle cells as models to characterize the properties of GLUT4-containing vesicles. GLUT4 is a continuously recycling molecule that cycles between intracellular pools and the plasma membrane (131). The stability of GLUT4 (half-life: ~48h) allows these cycles to occur
repeatedly over several days (132). Built-in within this cycle is the ability of GLUT4 to be sorted into a sequestered pool referred to as GLUT4 storage vesicles (GSV), which only progress to the plasma membrane in response to insulin stimulation (133). This intracellular retention is partly mediated by the C-terminal TELEY motif of GLUT4 because deletion of this motif redistributes more GLUT4 vesicles into the recycling pool (134). At steady state, ~50% of GLUT4 resides in the constitutively recycling pool while the other half is sequestered in the GSV (135). Biochemically, GSV differ from the general recycling population because they do not contain transferrin receptor. Instead, the presence of vesicle-associated membrane protein 2 (VAMP2) and insulin-regulated aminopeptidase (IRAP) on the GSV marks this compartment (135,136). Despite inferring the existence of this specialized compartment of GLUT4 from kinetic and biochemical data, the precise localization of these GSV within the cell remains difficult to visualize.

Two models currently exist to explain how GLUT4 distribution is maintained between recycling and sequestered pools in adipocytes and muscles. In the static retention model, GLUT4 is stored in the GSV and does not actively exchange with the recycling pool (137). On the contrary, the dynamic recycling model states that GLUT4 in GSV slowly traverses to the recycling compartment so that over time all the available GLUT4 within the cell is accessible to the plasma membrane (138,139). In adipocytes, similar strategies were used in studies leading to the disparate conclusions in GLUT4 vesicle cycling pattern, and it was recently suggested that the difference may arise from the cell culture confluency and the precise method of measurement (140). While debates remain in adipocytes, dynamic recycling is the favoured model in muscle cells.
Nonetheless, the common theme remains to be that insulin promotes the recruitment of GLUT4 vesicles to the plasma membrane from the GSV.

1.6-1-II: Insulin signalling cascade leading to GLUT4 translocation

As for any recycling protein, the gain in surface GLUT4 induced by insulin could in theory arise from decreased internalization (endocytosis) or increased externalization (exocytosis) of GLUT4 molecules. When examining insulin’s effect on the endocytosis of GLUT4, a minor reduction in the rate of GLUT4 internalization was observed in adipocytes while no changes were reported in muscle cells (141). It is therefore generally accepted that the major contribution to the insulin-stimulated gain in GLUT4 at the plasma membrane is the elevated rate of exocytosis of GLUT4-containing vesicles. For this reason, the signalling molecules activated by insulin to promote this exocytic step have been studied extensively with various cellular and animal knockout strategies. The detailed pathway is carefully reviewed in these articles (131,142,143) so a simplified version will be briefly described (Figure 1-6).
3.3

Figure 1-6: Insulin-stimulated signalling cascade leading to GLUT4 traffic in muscle cells. Upon binding of insulin to its receptor at the plasma membrane, autophosphorylation of its cytosolic tyrosine residues trigger the recruitment and subsequent phosphorylation of IRS-1. Tyrosine phosphorylated IRS-1 acts as a scaffold that interacts with PI3K, which leads to the membrane production of \( \text{PI}(3,4,5)\text{P}_3 \). At this point, the signals bifurcate into two independent pathways characterized by the activation of Akt and Rac-1. While Akt controls the activation of Rabs via inhibition of AS160, Rac-1 regulates the dynamics of actin remodelling following insulin stimulation in muscle cells. Altogether, these signalling molecules initiate the mobilization of GLUT4 vesicles from the GSV to the plasma membrane to achieve the increase in GLUT4 translocation response.

1.6-1-II-A: IR->IRS1/2->PI3K

It is well established that the insulin receptor (IR) undergoes autophosphorylation on its cytosolic tyrosine residues upon binding of insulin. The result of this is the subsequent recruitment and tyrosine phosphorylation of insulin-responsive substrate 1/2 (IRS-1/2) to amplify the signal. Differential contribution of IRS-1 and IRS-2 to insulin signalling has been verified by using both siRNA-mediated down-regulation in cells and genetic knockout strategies in animals (144). In muscle cells, disruption of IRS-1, but
not IRS-2, significantly impairs the insulin-mediated GLUT4 translocation and glucose uptake and concomitant down-regulation of IRS-1 and IRS-2 produces no additional deleterious effect (145). However, ablation of IRS-2 creates a greater reduction in the activity of mitogen-activated protein kinases responsible for cell proliferation. These observations suggest that IRS-1 is differentially programmed to carry out insulin-induced glucose uptake in muscles while IRS-2 governs mitogenic responses.

Functioning as a scaffold, phosphorylated IRS-1 binds the regulatory p85 subunit of phosphatidylinositol-3-kinase (PI3K), which leads to the activation of its catalytic p110 subunit. PI3K phosphorylates the third hydroxyl position of the inositol ring on \( \text{PI}(4,5)_2 \) to produce plasma membrane \( \text{PI}(3,4,5)_3 \). Experiments using PI3K inhibitors, including wortmannin and LY294002, and overexpression of p85 mutant that does not interact with p110 not only block the generation of \( \text{PI}(3,4,5)_3 \) but also significantly reduce insulin-stimulated GLUT4 translocation and glucose uptake (146,147). On the contrary, overexpression of constitutively active PI3K stimulates GLUT4 surface level independent of insulin (148). These findings validate the importance of PI3K-dependent membrane \( \text{PI}(3,4,5)_3 \) production in insulin signal cascade leading to GLUT4 mobilization.

1.6-1-II-B: Akt->AS160->Rabs

The presence of \( \text{PI}(3,4,5)_3 \) at the plasma membrane can channel activation of a number of downstream molecules notably Akt. Akt is a serine/threonine kinase that becomes active by phosphorylation at its activation loop (Thr308) and regulatory site (Ser473) (149,150). Insulin fulfills this requirement by recruiting Akt near the membrane through association with the plasma membrane \( \text{PI}(3,4,5)_3 \) via its PH domain, and inducing the subsequent phosphorylation of Thr308 and Ser473 by PDK1 and mTOR2,
respectively. The functional necessity of Akt in insulin-mediated GLUT4 translocation and glucose uptake was first demonstrated by overexpression of dominant negative mutant of Akt (147). It was later refined with additional knock out studies in cells and mice that demonstrated the specificity of Akt2 isoform, rather than the Akt1 and Akt3, in controlling the insulin-induced regulatory effect on glucose uptake (151).

Although Akt has a number of substrates to exert its biological functions (152), the exact downstream target required for insulin-stimulated GLUT4 traffic remained a mystery until the discovery of Akt substrate of 160kDa (AS160). AS160 (also known as TBC1D4) is phosphorylated by Akt in response to insulin and any decrease in its phosphorylation status correlates with impaired insulin-induced glucose uptake (153). Functionally, AS160 serves to retain GLUT4 intracellularly in the steady state. This action is dependent on its Rab GAP domain, in which the GTPase promoting activity is turned off upon phosphorylation. This is predicted to result in elevation of GTP-bound Rabs to liberate GLUT4 vesicles towards the plasma membrane (154). This observation led to the detailed mining of the Rabs controlled by AS160 following insulin stimulation. 

_In vitro_ experiments with purified GAP domain of AS160 first revealed its activity towards selective Rabs including Rabs 2A, 8A, 8B, 10 and 14 (155). These targets were subsequently examined in cell cultures via siRNA-mediated knockdown to validate the action of AS160 towards selective Rabs and the participation of Rabs in insulin-mediated GLUT4 traffic. While Rab10 is found to be downstream of AS160 in adipocytes and required for GLUT4 translocation induced by insulin, Rab8a and Rab13, but not Rab10, are necessary to carry out the same function in muscle cells (155-158). Accordingly, insulin leads to the activation (GTP-loading) of Rab8a and Rab13 in
muscle cells but Rab10 activation in adipocytes remains to be demonstrated (156). Ongoing research aimed at identifying the effectors of these activated Rabs is beginning to reveal the participation of motor proteins that may promote GLUT4 mobilization to and/or fusion with the plasma membrane (157,159).

1.6-1-III: Rho-induced actin regulation in GLUT4 traffic

Given actin’s ability to control a vast amount of cellular processes including vesicle traffic, an obligatory question over the years has been whether F-actin participates in the insulin-mediated GLUT4 translocation. This hypothesis was first tested using cultured L6 myotubes, where it was shown that acute stimulation of insulin causes a robust cortical actin reorganization (160). In the basal state, staining of F-actin by rhodamine phalloidin reveals dense longitudinal stress fiber spanning across myotubes. Following 30 min of insulin stimulation, a dramatic rearrangement of F-actin occurs which is marked by the dorsal accumulation of F-actin aggregates at discrete regions of the cell. Time course analysis later illustrated that insulin is capable of inducing this morphological change in F-actin by as early as 3 min (161). Although the remodelled F-actin can still be observed after 30 min of insulin treatment, the greatest magnitude of reorganization is achieved by 10 min, which has become the standard time point to visualize insulin-mediated actin remodelling in muscle cells. Similar cortical actin rearrangement can also be reproduced in undifferentiated myoblasts (162). Representatives of actin remodelling in myoblasts and myotubes following 10 min of insulin stimulation are illustrated in Figure 1-7.
Figure 1-7: Insulin-induced actin remodelling in myoblasts and myotubes. L6 myoblasts and differentiated L6 myotubes were stimulated with 100nM insulin for 10 min before being fixed and examined for actin reorganization. In the basal state, predominant F-actin staining detected by rhodamine phalloidin is cellular stress fibers. Upon stimulation by insulin, F-actin undergoes significant rearrangement to generate cortical remodelled actin (indicated by the arrows). Bar = 20µm.

To decipher the contribution of remodelled actin to the overall insulin response in muscle cells, Tsakiridis et al. pretreated myotubes with actin disrupting agent cytochalasin D (CD), which prevents actin polymerization by binding the barbed ends and promotes F-actin disassembly, and blocked insulin-responsive actin rearrangement (160). Interestingly, the corresponding GLUT4 translocation and glucose uptake induced by insulin were significantly decreased following CD treatment while the basal response was not affected. More importantly, under this actin depolymerizing condition, phosphorylation of IRS-1 and binding of PI3K to IRS-1 in response to insulin were not altered. These observations suggest the potential of insulin-elicited changes in actin as an additional step in regulating GLUT4 traffic in muscle cells. Similar reliance on actin was also observed in adipocytes (163).

Results from the application of different versions of actin disrupting agents, including CD, latrunculin B (LB) and jasplakinolide, in cultured and isolated primary adipocytes and muscle tissue reinforce the concept that insulin-induced actin dynamics is required for the stimulated glucose uptake or GLUT4 traffic to the plasma membrane.
(161,163-168). However, the drugs used in the above studies prevent all forms of F-actin assembly of all kinds within the cells so that it becomes difficult to assess whether the contribution of actin toward GLUT4 traffic arises from the insulin-induced remodelled cortical actin, or from the participation of longitudinal stress fibers, or other forms of actin filaments present even before insulin stimulation. We hypothesize that a useful strategy to distinguish between these possibilities would be to identify the molecules that govern the insulin-dependent actin remodelling.

Visually, the insulin-stimulated actin reorganization in both adipocytes and muscle cells resembles the morphological changes seen with active Rho GTPases, like that induced by Cdc42 and Rac, which makes Rho GTPases the most likely candidate in regulating this response. The earliest attempt to address this hypothesis came with experiments using Clostridium difficile toxin B, which is a general Rho GTPase inhibitory toxin (164). Treatment of adipocytes with Clostridium difficile toxin B not only blocks insulin-induced actin remodelling but also prevents the subsequent GLUT4 translocation (164). This observation laid the groundwork that begins the search for the member of the Rho GTPases mediating this actin reorganization.

1.6-1-III-A: TC-10 in adipocytes?

Although insulin stimulation causes a rapid remodelling of cortical actin filaments in both adipocytes and muscle cells, the dependency on Rho GTPases for this response differs between the two cell types. For adipocytes, it has been proposed that TC-10, a Cdc42 family GTPase, becomes activated by insulin in a PI3K-independent manner, and that disruption of TC-10 with dominant negative mutants or siRNA impairs insulin-induced actin remodelling (169,170). The activity of TC-10 is driven by the
recruitment of C3G, a GEF for TC-10, to the plasma membrane through a cascade of adaptor proteins (171). However, the proposition of TC-10 in insulin-stimulated actin remodelling has been greatly debated due to conflicting results in adipocytes. The inhibitory effect of TC-10 on actin is independent of its nucleotide status and down-regulation of adaptor components necessary for C3G-induced TC-10 activation fails to reduce insulin-induced glucose uptake (172,173). More recent evidence suggests Rac might be the Rho GTPase responsible for the actin-mediated action on GLUT4 traffic in adipocytes (174). The precise molecule remains to be clarified.

1.6-1-III-B: Rac-1 in muscles

Despite TC-10 also being activated in muscle cells, expression of its dominant negative form did not prevent the formation of insulin-dependent actin remodelling, heralding the participation of other Rho GTPase in the actin morphology change (175). Due to the resemblance of remodelled actin to that of Rac-mediated lamellipodia in migrating cells, it was hypothesized that Rac could be the regulator of insulin-induced actin reorganization. Indeed, insulin activates Rac-1 as early as 1 min and its GTP-loaded status peaks by 10 min in muscle cells (175,176). Functionally, both DN-Rac overexpression and down-regulation of endogenous Rac-1 via siRNA prevent the generation of remodelled actin in response to insulin and impair GLUT4 translocation (161,177). No alteration in insulin-induced actin morphology was observed with the overexpression of dominant negative (DN)-RhoA or DN-Cdc42 in muscle cells, which reinforces the specificity of Rac1 in this response ((161), JeBailey and Klip, unpublished results). Unlike actin disrupting agents, perturbation of Rac-1 spares actin stress fiber dissolution, which allows the discrimination of insulin-induced remodelled actin as the F-
actin component participating in GLUT4 traffic. More recently, a skeletal muscle-specific Rac-1 knockout mouse models have been generated and verified the importance of Rac-1 in insulin-mediated GLUT4 translocation to the plasma membrane *in vivo* (178,179).

The most common mechanisms to activate Rho GTPase are the activation of their GEFs or inhibition of their GAPs. Little is known about the GEF and GAP that might participate in GTP loading of Rac-1 by insulin in muscle cells. However, evidence points to Rac1 being downstream of PI3K because wortmannin prevents both the formation of remodelled actin and the activation of Rac-1 following insulin stimulation (175). This observation supports the idea of Rac-GEF participation as many Rac-GEFs have been documented to be recruited and activated by membrane PI$_{(3,4,5)}$P$_3$ (180). FLJ00068 has been suggested to be the GEF for insulin-stimulated Rac-1 activation in muscle cells (181). Using a constitutively active form of this GEF, Ueda et al. reported an increase in Rac-1 activation and actin remodelling. On the other hand, siRNA-mediated down-regulation of FLJ00068 reduced GLUT4 translocation following insulin stimulation. However, this report did not provide a direct evidence of impaired Rac-1 activation or actin remodelling in FLJ00068 knock-down condition. More experiments are needed to clarify if this is the only Rac-GEF involved in the insulin signalling pathway.

1.6-1-IV: Akt and Rac-1 function independently but both lie downstream of PI3K

With Akt also acting as a downstream effector of PI3K, it was paramount to examine the interplay between Rac-1 and Akt and their respective contribution towards GLUT4 traffic in muscle cells. Interestingly, insulin-dependent activation of Rac-1 and
Akt is independent from each another. Inhibition of Akt with a DN-mutant or a chemical Akt inhibitor does not affect insulin-induced Rac-1 activation and actin remodelling (147,182) while normal Akt phosphorylation is preserved in cells with downregulated Rac-1 (177). These findings champion the concept that signal bifurcation into Rac-1 and Akt occurs downstream of PI3K upon insulin stimulation of muscle cells. However, a recent publication by Nozaki et al. using a different version of Akt inhibitor and Akt2 knockdown strategies reported an Akt-dependent activation of Rac-1 during insulin stimulation (183). The difference may be due to the varying inhibitory mechanism exerted by the two Akt inhibitors. Specifically, the initial signal bifurcation finding utilized Akt inhibitor VIII that selectively binds to the PH-domain of Akt to prevent its recruitment to plasma membrane $\text{P}_{3}$ leading to inactivation (184). In contrast, Akt inhibitor IV used by Nozaki et al. targets kinase upstream of Akt (185), which when inactivated could potentially affect other signalling components in addition to Akt. Furthermore, Nozaki et al. relied on a 2h pretreatment with their Akt inhibitor, which increases the chances of non-specific effects, in order to observe the decrease in insulin-stimulated Rac-1 activation while the earlier report utilized only 30 min of acute treatment. While evidence of an intact insulin-induced remodelled actin following Akt inhibitor VIII treatment serves as another indicator for signal bifurcation between Akt and Rac-1, this key Rac-dependent process was never tested in Nozaki’s report. In addition, the effect of Akt2 knockdown on insulin-stimulated Rac-1 activation could have been easily demonstrated in a population assay by applying the gold-standard PAK-mediated Rac-GTP pulldown method to detect endogenous Rac activation. However, Nozaki et al. chose to visualize this effect with a less convincing and scarcely used overlay single cell.
assay, which requires immunofluorescent detection of exogenously expressed WT-Rac in its GTP-bound form by incubating the cells with purified PAK. Therefore, it is extremely important to reassess the contribution of Akt to Rac-1 activation during insulin stimulation. Nonetheless, impairing either Akt or Rac-1 reduces insulin-triggered GLUT4 translocation, supporting a contribution from both molecules to the overall process.

1.6-1-V: Functions of insulin-induced remodelled actin in GLUT4 traffic

Although it is undeniable that a Rho GTPase-driven actin dynamics process participates in GLUT4 traffic, the precise mechanism whereby the remodelled actin assists in the mobilization and fusion of GLUT4-containing vesicle to the plasma membrane remains elusive. Several hypotheses have been proposed including tethering of GLUT4 vesicles, being a scaffold for signalling molecules, and serving as tracks for mobility (Figure 1-8).
**Figure 1-8: Proposed functions of cortical remodelled actin in insulin-mediated GLUT4 traffic in muscle cells.** Rac-dependent signals trigger a dynamic actin remodelling zone beneath the plasma membrane following insulin stimulation. This area rich in remodelled actin has been hypothesized to act as tracks for myosinV-mediated GLUT4 vesicle movement, tethering zone for GLUT4 vesicles, and insulin signalling molecules to promote the eventual fusion with the plasma membrane.

1.6-1-V-A: Tethering of GLUT4 vesicles

Using both electron and immunofluorescence microscopy, GLUT4 vesicles can be observed at the site of remodelled actin following insulin stimulation (161,165,166). The resulting proximity of GLUT4 vesicles to the plasma membrane could ease the spatial barrier of the subsequent fusion via interaction with the soluble NSF attachment protein receptor (SNARE) proteins required for GLUT4 vesicle fusion with the membrane (186). This model is supported by the findings that disruption of cortical actin with DN-Rac or LB prevented the insulin-induced accumulation of GLUT4 beneath the plasma membrane of muscle cells (187). In searching for the molecular link that would anchor GLUT4 vesicles to the F-actin network, the actin bundling protein α-actinin-4, was found to co-immunoprecipitate with GLUT4 in an insulin-dependent manner (188,189). Through its simultaneous interactions with F-actin and GLUT4, α-actinin-4 could position GLUT4 vesicles at the site of remodelled actin, and indeed siRNA to α-actinin-4 prevents the accumulation of GLUT4 vesicles in the actin meshwork and results in reduced GLUT4 insertion to the plasma membrane (188). This proposed molecular bridge exerted by α-actinin-4 supports the concept that the remodelled cortical actin serves as a tether of GLUT4 vesicles in muscle cells.

Myosin1c (myo1c) has also emerged as a candidate linking GLUT4 vesicles to the cortical actin. Myo1c interacts with actin filaments and the plasma membrane. Immunofluorescence microscopy and immunoprecipitation data also confirm its direct
interaction with GLUT4 but the association is not altered by insulin stimulation (190,191). Total internal reflection fluorescence (TIRF) microscopy experiments in muscle cells revealed that myo1c decreases the mobility of GLUT4 vesicles at the cell cortex following insulin stimulation to stabilize their position for subsequent fusion events (191). Without perturbation of the remodelled actin, down-regulation of myo1c or transfection of a myo1c mutant lacking the actin binding domain impairs this immobilization step, which results in compromised GLUT4 translocation in muscle cells.

This tethering concept is also showcased in adipocytes where LB treatment causes the loss of cortical remodelled actin and increases the number of mobile GLUT4 vesicles beneath the plasma membrane (192). However, other reports also in adipocytes showed the opposite phenotype in which disruption of actin reduces fusion with the membrane without affecting insulin-stimulated submembrane accumulation of GLUT4 vesicles (193,194). The discrepancies between the above studies may relate to differences in LB treatment time and concentration. Similar TIRF experiments in muscle cells treated with LB would clarify any cell difference in the actin-dependent tethering model.

1.6-1-V-B: Scaffold for signalling molecules

To spatially and temporally regulate GLUT4 traffic, the remodelled cortical actin could also act as a scaffold to enrich signalling molecules near the plasma membrane (162). In addition to GLUT4 vesicle presence in the F-actin meshwork, PI3K, Akt, and Rabs have also been detected in the cortical region of remodelled actin following insulin stimulation in muscle cells (156,161,162). Their close proximity to GLUT4 vesicles could form a hub for signal transduction of Akt->AS160->Rab as Rabs are found to associate
with GLUT4 vesicles (155). This would result in more efficient GLUT4 traffic at the plasma membrane. Recent analysis using a photo-switchable GLUT4 construct identified defined clusters of GLUT4 at the plasma membrane in the steady state (195). In addition to increasing the arrival of GLUT4 vesicle, insulin stimulation also enhances the lateral dispersion of GLUT4 away from its membrane clusters to become monomers. Although the molecules controlling the formation and the dispersion of GLUT4 clusters are not yet identified, it could be speculated that actin-dependent signalling hubs would enrich these proteins at the GLUT4 clusters to enhance their lateral dispersion.

1.6-1-V-C: Tracks for GLUT4 vesicles

F-actin can serve as tracks for the motor protein myosin. Among the myosin family members, myosin5 (myo5) is a processive motor with cargo carrying capacity that “walks” on actin filaments (196). Indeed, myo5 can be found associated with F-actin following insulin stimulation in adipocytes (197). siRNA to myo5 or overexpression of a myo5a DN-mutant causes mislocalization of GLUT4 vesicles and reduces translocation elicited by insulin (157,197). Interestingly, Rab8a in muscle cells can directly interact with myo5a in an insulin-dependent manner (Yi and Klip, unpublished results). This provides a mechanistic explanation for how Rab molecules mobilize GLUT4 vesicles in response to insulin. However, the reliance on F-actin for mobility is most likely restricted to short range movement close to the cell cortex where remodelled actin is formed because evidence supports microtubule rather than actin participating in long range transport (198,199).

1.6-1-VI: Rac-dependent actin remodelling during insulin resistance in muscle cells
The hallmark of insulin resistance is the inability of tissues to respond to insulin following elevation in blood glucose. With skeletal muscles accounting for ~90% of postprandial glucose absorption, one of the major contributors to this pathological state is the decreased GLUT4 traffic to the plasma membrane following insulin stimulation, which leads to impaired glucose uptake by skeletal muscles (200). The development of insulin resistance is multifactorial but the most common cause in the developed world is obesity. Tissues in obese individuals are exposed to excess lipids and are chronically stressed by low-grade inflammation arising from activated immune cells (201,202). Experimental manipulations aimed to mimic these obese cellular environments by addition of excess saturated fatty acid and cytokines produced by activated immune cells collectively attribute the defect in GLUT4 translocation and glucose uptake to the partial reduction in insulin-induced Akt activation (203-207). However, studies using wortmannin and Akt inhibitor reveal that minimal Akt activity is required for maximal GLUT4 translocation response (208-210). This would suggest that the ~50% inhibition of Akt typically observed in these insulin resistance models is unlikely to be the sole reason for the reduction in GLUT4 translocation and glucose uptake.

Because Akt and Rac-1 function independently of one another and both contribute to the integrity of GLUT4 traffic in muscle cells, combined impairment in Akt and Rac-1 synergize to cause the observed GLUT4 traffic defect. Part of the detrimental effect of saturated fatty acid palmitate oversupply is the build-up of intracellular metabolites such as ceramide and mitochondrial overproduction of reactive oxygen species (ROS) (211,212). Replicating these stresses via cell-permeable C2-ceramide and ROS-producing glucose oxidase significantly inhibited insulin-induced Rac-1
activation and actin remodelling in muscle cells (177). Notably, Rac-1 activation was more sensitive to these stress challenges compared to Akt. Additionally, high insulin and glucose mirroring the hyperglycemic/hyperinsulinemic state of insulin resistance also prevents the formation of remodelled actin and GLUT4 traffic following insulin stimulation (145). This demonstrates the contribution of defective Rac-1 activation and defective Rac-1-dependent actin reorganization to the development of insulin resistance. Indeed, recent mouse and human studies using acute (intralipid infusion) and chronic (obesity) insulin resistance confirm the decrease in Rac-1-dependent signals and pinpoint to Rac-1 as an important regulator of insulin-dependent GLUT4 traffic and glucose uptake in vivo (179).

1.7: RATIONALE AND HYPOTHESES

Given the importance of skeletal muscles in insulin-mediated glucose homeostasis and the essence of GLUT4 traffic in achieving this response, it is imperative to identify the complete molecular cues that trigger the net gain in GLUT4 at the muscle cell surface. With a thorough understanding of this GLUT4 recruitment process from intracellular compartments to the plasma membrane, it will become possible to identify the individual defects underlying insulin resistance in diverse cellular and organismic settings. This would potentially allow for the development of treatment strategies designed specifically to bypass or alleviate the signal transmission breakage. With Rac-1 and Rac-1-dependent actin remodelling emerging as critical components of insulin-stimulated GLUT4 traffic in muscles, detailed experimental emphasis on Rac-1 and remodelled actin is needed.
Although Rac-1 is responsible for initiating actin reorganization in response to insulin, the downstream effectors of Rac-1 that contribute to this dynamic actin remodelling phenotype are still unresolved. Uncovering these effectors would not only decipher how the remodelled actin is generated but also enable the design of molecular strategies to restore the actin dynamics. By examining the changes in remodelled actin and GLUT4 traffic after perturbation of Rac-1-dependent effectors, we would better understand the exact role of remodelled actin and refine the previously proposed tether, scaffold, and track models. Studies looking at similar Rac-induced actin structures in migrating cells reveal that Rac-mediated actin remodelling requires a balance between polymerization and depolymerization (94). This process is commonly associated with the Arp2/3-dependent branching activity and the cofilin severing/depolymerizing potential. However, it is unclear whether Arp2/3 and cofilin are the main drivers of actin dynamics in response to insulin in muscle cells.

Increasing evidence from mouse and human models has confirmed the initial findings about the functional impact of Rac-1 in insulin signalling (178,179). In their analysis of insulin resistance, Rac-1 was consistently found to have reduced activity coinciding with diminished GLUT4 traffic. As a result, it becomes intriguing to examine whether increasing Rac-1 levels or Rac-1 activity would exert any positive signalling effect towards insulin-responsive GLUT4 traffic and counteract the impact of insulin resistance-causing conditions. Of interest, one study reported that constitutive active (CA)-Rac overexpression can on its own effect in surface GLUT4 (181). However, the molecular mechanism behind this phenotype is unknown.

To address these two questions, I hypothesize the following:
1. The insulin-induced Rac-1-dependent actin remodelling requires the actin polymerizing factor, Arp2/3, and the depolymerizing factor, coflin, in order to achieve the dynamic remodelling of cortical actin filaments to facilitate GLUT4 traffic.

2. Increasing Rac-1 activity in muscle cells will impart a beneficial effect on GLUT4 traffic that could enhance GLUT4 translocation and confer protection from its defect in insulin resistance-causing conditions.

I will use the established L6 GLUT4myc cell line with various molecular manipulations to examine these two hypotheses in chapters 3 and 4.
CHAPTER 2: Materials and Methods
2.1: Materials

2.1-1: Cell culture reagents

α-minimum essential medium (αMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin, antibiotics were from Wisent (Montreal, QC).

2.1-2: General reagents

Protease inhibitor cocktail (PIC), NaF, Na$_3$VO$_4$, rapamycin, IPA-3, and DMSO (high pressure liquid chromatography grade) were from Sigma-Aldrich (St. Louis, MO). Human insulin was purchased from Eli Lilly (Indianapolis, IN). SuperScript® VILO™ cDNA kit, Trizol, Taq PCR DNA polymerase, and rhodamine-phalloidin was from Invitrogen (Grand Island, NY). Akti inhibitor 1/2 (Akti1/2), compound C (CC), Tween-20, NP-40, and latrunculin B (LB) were from Calbiochem (Gibbstown, NJ). Wortmannin and C2-ceramide were from Enzo Life Science (Farmingdale, NY).

2.1-3: Antibodies

Monoclonal anti-P-tyrosine antibody, polyclonal anti-myc antibody (A-14), anti-Tiam1, anti-Vav-2, anti-GDIα, anti-P-GDIα, and anti-p34-ARC were from Santa Cruz. Polyclonal anti-phosphorylated cofilin-1, anti-Akt, anti-phosphorylated Akt (Ser473), Akt(Thr308), anti-phosphorylated Akt substrate (PAS), and anti-phosphorylated LIMK1 antibodies were from Cell Signaling Technology (Danvers, MA). Polyclonal anti-Arp3 was from BD Biosciences. Polyclonal anti-slingshot-1 (anti-SSH1) was from ECM Biosciences. Affinity purified antibodies against P-AC and cofilin-1 were as described (213,214). Monoclonal anti-ACTN1 (mouse IgM isotype, clone BM-75.2), anti-β-actin, polyclonal anti-LIMK1, monoclonal anti-FLAG antibody were from Sigma-Aldrich. Polyclonal anti-GFP antibody was from Abcam Inc (Cambridge, MA). Monoclonal P-
IRS-1(307) antibody was from EMD Millipore (Billerica, MA). Monoclonal anti-HA antibody was purchased from Covance Inc (Princeton, NJ). Cy3 and Cy5 conjugated donkey-anti-rabbit and donkey-anti-mouse secondary antibodies and horseradish-peroxidase (HRP) conjugated goat-anti-rabbit, goat-anti-mouse, and rabbit-anti-goat secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Alexa 488-conjugated goat anti-mouse and anti-rabbit IgG antibodies were from Invitrogen.

2.1-4: cDNAs

Green fluorescent protein (GFP)-tagged Dictyostelium discoideum Arp3 was kindly provided by Dr. Sergio Grinstein (University of Toronto, Toronto, ON, CA). GFP-tagged wildtype and S3E Xenopus cofilin were generated in the laboratory of Dr. J. Bamburg (215).

The cDNA constructs (Lyn-FRB, YF, YF-CA-Rac, YF-Tiam1, and YF-DN-Rac) used for rapamycin-induced heterodimerization have been previously described and were generously provided by Dr. Mary Teruel and Dr. Tobias Meyer (216). WT-Rac- and GFP-CA-Rac were provided by Dr. Mark R. Philips. HA-IRS1 was provided by Dr. Maria Rozakis. FLAG-CA-JNK1 was provided by Dr. Gokhan Hotamisligil (217). Tiam1-GFP was provided by Dr. Sergio Grinstein. CFP tagged WT-Cdc42 and CA-Cdc42 were provided by Dr. John Brumell.

2.1-5: siRNAs

Small inhibitory RNAs (siRNAs) targeted against p34 (AAGGAACCTTCAGGCACACGGA), Arp3 (GAAAGCGTGGATGACCTATT) (218), cofilin-1 (AAGGTGTTCAATGAGATGAAA), LIMK1
(AAGGAATGTGCCGCTGGCAGATT), slingshot-1 (AAGAACTGAGCGTCTCATTAA),
Tiam1 (CTGCCGGAATTGGTGTGCGA), Vav-2 (CCAGATGTACACGTTTGACAA), and
non-related control (AATAAGGCTATGAAGAGATAC) were purchased from Qiagen,
prepared as 20uM stock in RNAse-free water, and are here named, respectively, sip34,
siArp3, siCofilin, siLIMK, siSSH1, siTiam1, siVav-2, and siNR.

2.1-6: PCR primers
Primers are designed using Primer3 software (http://frodo.wi.mit.edu/) and ordered from
ACGT Inc. (Toronto, Ontario).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Expected size (bp)</th>
</tr>
</thead>
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<td>tgcaataacgccataaaca</td>
<td>254</td>
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<tr>
<td>Vav-2</td>
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<td>tcatgtctccgcttgcag</td>
<td>242</td>
</tr>
<tr>
<td>Vav-3</td>
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<td>tggcgactttgtactgtg</td>
<td>405</td>
</tr>
<tr>
<td>Tiam1</td>
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<td>296</td>
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<tr>
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<td>tgatgagctgggtagggtctc</td>
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<tr>
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<td>gtttggcagcagctttttcc</td>
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</tr>
<tr>
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<tr>
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</tbody>
</table>

2.2: Experimental protocols
2.2-1: Growing L6 myoblasts
Rat L6 muscle cells expressing GLUT4 with an exofacial myc epitope (L6-GLUT4myc) were cultured as described previously (219). Myoblasts were cultured in αMEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) antibiotics in a humidified incubator with 5% CO₂ at 37 °C. The myoblasts were split every 48h to prevent aggregation of the cells.

2.2-2: Cell treatments

For experiments looking at insulin-dependent GLUT4 translocation in L6GLUT4myc myoblasts, cells were serum starved in αMEM for 3h prior to 100nM of insulin stimulation for 10 min. For experiments with inhibitors, various concentration and pretreatment time in serum free αMEM were applied prior to 10 min insulin stimulation in the presence of the compound. The conditions are as follows:

Table 2-2: Summary of compounds and their treatment concentration and time.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Pretreatment time</th>
<th>Insulin stimulation with compound</th>
<th>Total treatment time</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>250nM (for confluent myoblasts)</td>
<td>20 min</td>
<td>10 min</td>
<td>30 min</td>
</tr>
<tr>
<td></td>
<td>200nM (for sparse single cell)</td>
<td>20 min</td>
<td>10 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>100nM</td>
<td>20 min</td>
<td>10 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Akti1/2</td>
<td>10µM</td>
<td>30 min</td>
<td>10 min</td>
<td>40 min</td>
</tr>
<tr>
<td>Compound C</td>
<td>10µM</td>
<td>30 min</td>
<td>10 min</td>
<td>40 min</td>
</tr>
<tr>
<td>C2-ceramide</td>
<td>50µM</td>
<td>2h</td>
<td>10 min</td>
<td>2h 10min</td>
</tr>
<tr>
<td>IPA-3</td>
<td>30uM</td>
<td>30 min</td>
<td>10 min</td>
<td>40 min</td>
</tr>
</tbody>
</table>

2.2-3: cDNA transfection

Transfection of cDNA was performed with Lipofectamine 2000 (Invitrogen) or Fugene HD (Promega) according to the manufacture’s protocol. For immunofluorescence
experiments, myoblasts were seeded on 12mm coverslip in 12 well plate a day before the transfection so that the cell density is ~50% on the day of transfection. cDNA-lipofectamine was mixed at 0.5µg-to-1µL or 1µg-to-2µL ratio in 100µL serum free media, incubated at room temperature for 25 min, and mixture was subsequently applied to the cells in serum free media for 5h before washing 2x with PBS and exchanging to fresh growth media. Cells were allowed to recover overnight before experiments in the next day. For experiments with immunoprecipitation of transiently expressed proteins, myoblasts were seeded on 10cm dishes a day before so they will be ~70% confluent on the day of the transfection. cDNA-Fugene was mixed at 4µg-to-12µL ratio in 200µL serum free media, incubated at room temperature for 25 min, and the mixture was treated with the cells in growth media without antibiotics for 24h to achieve higher transfection efficiency. After 24h, cells were washed 2x with PBS and continued with the immunoprecipitation experiments. Maximal transfection efficiency in L6 myoblasts is ~20-30%.

2.2-4: siRNA transfection

For siRNA experiments, myoblasts were seeded a day before so that a cell density of ~70% is reached on the day of transfection. Transfection of siRNAs was achieved using the calcium phosphate-based CellPhect Transfection Kit according to the manufactured protocol (GE Healthcare Bio-Sciences). 200nM siRNA-calcium phosphate precipitates were removed 12-16 h after addition and cells were maintained for 72 h until experimentation. Alternatively, siRNA mixture was prepared with JetPrime from Polyplus Transfection Inc. (Illkirch, France). For each well of a 24 well plate, 3µL of 20µM siRNA stock was mixed with 50µL of JetPrime buffer. 1.5µL of JetPrime reagent
was subsequently added to the mixture and vortexed for 10 sec. After incubation at room temperature for 25 min, the siRNA mixture was added to the well containing growth media without antibiotics and incubated for 24h. Following the 24h treatment, cells were washed 2x with PBS, replaced with fresh growth media, and maintained for another 48h before conducting experiments. For each well in 12 well and 6 well format, 6µL of siRNA+100µL JetPrime buffer+3µL JetPrime reagent and 12µL of siRNA+200µL JetPrime buffer+6µL JetPrime reagent formulation was used respectively.

2.2.5: RNA isolation

RNA was isolated using Trizol (Invitrogen). Myoblasts were grown to confluency in 6 well plate. After 2x quick washes with cold PBS, 1mL of Trizol (1mL per 10cm² ratio) was added to each well with the plate on ice. The cell lysate were passed through P1000 pipette 10x before transferring to an eppendorf tube followed by 5 min incubation at room temperature. 0.2mL of chloroform was added to the homogenized samples (0.2mL chloroform per 1mL Trizol ratio) and the solution was vortexed for 15 sec before resting at room temperature for another 2 min. Samples were subsequently centrifuged at 12000 x g for 15 min at 4°C to separate mixture into lower red phase (phenol-chloroform), interphase, and colorless aqueous phase at the top. The aqueous top phase is carefully transferred to a fresh tube. To precipitate RNA, 0.5mL of isopropyl alcohol was added to the aqueous phase, vortexed, and incubated at room temperature for 10 min. The mixture was centrifuged at 12000 x g for 10 min at 4°C to form RNA precipitate at the bottom of the tube. After removal of supernatant, RNA pellet was washed 2x with 1mL of 75% ethanol and centrifugation at 7500 x g for 5 min at 4°C. The RNA pellet inside the tube was air-dried completely before redissolved in 20µL DEPC-treated water. The concentration of each samples were measured by A₂₆₀/A₂₈₀ ratio.
2.2-6: cDNA generation from purified RNA

Reverse-transcription-dependent cDNA generation was completed using SuperScript® VILO™ cDNA kit (Invitrogen). 2µg of RNA was mixed with 4µL of 5X VILO™ Reaction Mix, 2µL of 10X SuperScript® Enzyme Mix, and topped with DEPC-treated water to 20µL. The mixture was gently mixed and incubated at 25°C for 10 min. Reaction was started by placing the tube at 42°C for 60 min followed by termination at 85°C for 5 min.

2.2-7: PCR

PCR reaction was achieved using Taq PCR DNA polymerase (Invitrogen). A 20µL PCR mixture was created as follows: 2µL of 10X PCR buffer, 0.5µL of 50mM MgCl₂, 0.4µL of 10mM dNTP mix, 0.4µL of 10µM forward primer, 0.4µL of 10µM reverse primer, 0.1µL of Taq DNA polymerase, 0.4µL of cDNA, and topped up to 20µL with autoclaved distilled water. The mixture was heated at 95°C for 2 min for denaturation of template and activation of enzyme. 30 cycles of PCR amplification was performed as follows for semi-quantitative readouts: denature at 95°C for 30 sec, anneal at 58°C for 40 sec, and extend at 72°C for 1 minute per kb. The results were analyzed by agarose gel electrophoresis with the appropriate DNA ladder.

2.2-8: Cell lysates, immunoprecipitation and immunoblotting

After transfection and experimental treatments, cells were washed quickly 2x with cold PBS and lysed with 1% (vol/vol) Triton X-100 in PBS containing 1:500 PIC, 10mM NaF, and 10mM Na₃VO₄. Lysates were passed through a 29-gauge syringe needle ten times, and supernatants were collected by a 10 min spin at 12,000x g. For standard immunoblotting, concentration of the supernatant was measured to ensure equal
loading of samples for comparison. Typically, 10-15µg of proteins was prepared with 2x Laemmli sample buffer for immunoblotting.

For immunoprecipitation, supernatant was subsequently incubated with protein G beads conjugated to 1-2µg of epitope antibody at 4°C for at least 4h before subjecting to 3x washes with 0.1% Triton X-100 followed by a last wash with regular PBS without detergent. Immunoprecipitated protein samples were eluted with 2x Laemmli sample buffer. All protein samples are subsequently resolved by 7, 10, or 13% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA) at 100V for 2h. Membranes were subsequently blocked with 3% (vol/vol) BSA in Tris-buffered saline (500mM Tris-base, 150mM NaCl, 0.5% (vol/vol) Tween-20, 0.5% (vol/vol) NP-40, pH=7.5) for 1h and immunoblotted with respective antibodies (1:1000) overnight at 4°C. Primary antibodies were detected with the appropriate HRP-conjugated species-specific IgG secondary antibodies (1:10000) at room temperature for 1h. Immunoblotting was completed with Western Lightning Chemiluminescence Reagent Plus and HyBlot CL autoradiography film from Denville Scientific (Denville, NJ).

2.2-9: Cell-surface GLUT4myc and total GLUT4myc detection by immunofluorescence microscopy

Immunofluorescence detection of surface GLUT4myc in adhered L6 myoblasts was performed as previously described (166). Following 3 h serum starvation, cells were stimulated with/without 100nM insulin for 10 min at 37°C. Cells were quickly washed twice with cold phosphate-buffered saline supplemented with calcium and magnesium (PBS+), fixed with 3% (vol/vol) paraformaldehyde, quenched with 0.1M glycine and blocked with 5% (vol/vol) milk. Surface GLUT4myc was stained by incubating anti-myc primary antibody followed by Cy2- or Cy3-coupled secondary
antibody. For co-staining of surface GLUT4myc and intracellular F-actin, surface GLUT4myc was labeled first prior to membrane permeabilization with 0.1% Triton X-100 for 3 min before subsequent F-actin staining with rhodamine phalloidin. For co-staining of tagged cDNA constructs, 3 min permeabilization with 0.1% Triton X-100 and detection with epitope antibody were applied after labeling of primary myc antibody. For measurement of rapamycin-induced gain in surface GLUT4myc, 10 min of 1µM rapamycin treatment was applied to cells transfected with respective components of rapamycin heterodimerization system. For total GLUT4myc detection, cells were permeabilized with 0.1% Triton X-100 for 15 min following the quenching step prior to blocking and labelling with anti-myc primary antibody. Fluorescence images were taken with a Zeiss LSM 510 laser-scanning confocal microscope (Thornwood, NY). With 63x objective, cells were scanned along the z-axis, single composite image (collapsed xy projection) of the optical cuts (1µm) per cell was assembled, and the pixel intensity of each cell (≥25 cells per condition) was quantified by ImageJ software.

2.2-10: Rounded-up myoblasts assay

Rounded-up myoblasts were generated as previously described (187). Regular adherent L6 myoblasts or cDNA transfected myoblasts were incubated in PBS without calcium and magnesium at 37°C for 10 min to allow detachment. After careful removal of PBS, cells were re-suspended in serum free media with/without insulin or rapamycin before attached on the coverslip for 10 min at 37°C followed by fixation. For staining of surface GLUT4myc in rounded-up myoblasts, standard surface detection protocol of surface GLUT4myc is employed as described above.

2.2-11: Detection and quantification of actin remodelling/polymerized dorsal actin
Following treatment with or without insulin for 10 min at 37 °C, adhered or rounded-up L6 myoblasts were fixed, quenched, permeabilized with 0.1% Triton X-100 in PBS for 3 min, blocked with 5% milk and stained with rhodamine phalloidin for F-actin. For rapamycin-triggered changes in F-actin, cDNA-transfected myoblasts were first treated with/without rapamycin for 10 min at 37°C before following the staining with rhodamine phalloidin. Cells were imaged by Zeiss LSM 510 laser scanning confocal microscope. Acquisition parameters were adjusted to minimize saturation of the signal. To quantify actin remodelling, the F-actin pixel intensity from compiled dorsal optical slices of basal and insulin-stimulated cells were analyzed with ImageJ software (>30 cells per condition). The optical slices quantified began from the outermost confocal fluorescent signal detected and continued towards the interior of the cell but skipping the last 2-3 optical slices dominated by actin stress fibers. By eliminating from the quantification the focal planes enriched in parallel arrays of stress fibers, which are still devoid of crisscrossed arrays of branched actin (vastly present in the top half of the cells towards the dorsal surface), we quantify cortical remodelling. The method may potentially underestimate this response, but not magnify it.

2.2-12: $^{125}$I-Transferrin recycling

The recycling of transferrin (Tf) was measured essentially as previously described (220). Following siRNA treatment and 3 h serum starvation, myoblasts were labeled with $^{125}$I-Tf (1µg/ml) for 30 min at 37°C. Cells were placed on ice, washed once with cold medium (α-MEM, 1% BSA, 20mM HEPES), once with cold acid solution (0.15M NaCl, 0.1M glycine, pH=3.0), and again with cold medium. Cells were then chased with medium containing 200µg/ml holo-Tf with/without insulin at 37°C and the
medium was collected at the indicated time points. Following another wash with cold medium, cells were scraped and lysed in 1M NaOH. Radioactivity in cell lysates (internalized Tf) and medium collected (externalized Tf) was measured in a gamma counter. Data for each time point were measured in triplicate. Tf recycling was expressed as the ratio of externalized Tf over internalized Tf. Data were corrected for non-specific binding of ¹²⁵I-Tf by the addition of 200µg/ml holo-Tf during the initial labeling.

2.2-13: Rac-1-GTP pulldown assay

Rac-1 activation assay was performed as previously described (175). For preparation of GST-CRIB beads, bacterial stock containing the GST-CRIB expression plasmid was inoculated in 10mL of LB containing 100µg/mL ampicillin and grown overnight at 37°C under rotation. The next day, 10mL of bacteria was added into a flask of fresh 250mL LB. Growth is continued at 37°C with rotation until the optical density at 600 (OD600) is reached between 0.4~0.6. At this point, a final concentration of 0.5µM IPTG was added to the flask to induce protein expression for 4h at 37°C with shaking. At the end of the induction, bacteria was spun down at 6000x g for 15 min and frozen at -80°C until purification with French press.

Frozen bacterial pellet was resuspended in cold STE buffer (10mM Tris, 150mM NaCl, 1mM EDTA) containing 1:500 PIC. The solution was passed through French press at 1000 psi 2x to liberate proteins by breaking bacterial membranes. Triton X-100 was added next to a final concentration of 1% vol/vol. The solution was mixed under rotation at 4°C for 30 min before spin down at 12000x g for 30 min. During the spin down, ~1mL of glutathione sepharose beads were washed 3x with STE buffer before the supernatant from the spin down was added to the beads. Incubation was permitted
at 4°C under rotation for 1h. The resulting conjugated beads were washed 3x with 15mL of STE buffer each. Final conjugated GST-CRIB beads were aliquoted and stored at -80°C.

To perform the active Rac-1 pulldown assay, myoblasts were grown to confluence on 10cm dishes before experiments. Cells were serum starved for 3h before stimulation with/without 100nM insulin for 10 min. After 2x wash with cold PBS, lysates were collected with MLB buffer (25mM Hepes, 150mM NaCl, 1% NP-40, 10mM MgCl2, 1mM EDTA, 2% glycerol) supplemented with 1:500 PIC+10mM NaF+10mM Na3VO4, span down at 12,000x g for 1 min, and incubated with GST-CRIB beads under rotation at 4°C for 45 min. After 3x washes with MLB followed by last wash with PBS without detergent, the pulldown proteins were eluted with 2x Laemmli sample buffer and resolved in 10% SDS-PAGE for immunoblotting for Rac-1. For Akti1/2 experiments, inhibitor or DMSO was applied in the last 1h of serum starvation. For Rac activation with GFP-WT-Rac or GFP-CA-Rac, myoblasts were transfected with the respective constructs for 24h before the experiments. The pulldown Rac-GTP was detected with GFP antibody.

2.2-14: 2D immunoblots

2D immunoblots were performed according to the previously published method (214). Cell extract with ~100µg of protein was placed in a 1.5mL tube and the protein was precipitated with chloroform/methanol. Protein pellets were air-dried and solubilized in 8M urea containing 2% IGEPAL CA-630 and 20 mM DTT. Before loading onto 7cm IPG strips for isoelectric focusing, IPG buffer (pH 3–10, Amersham Biosciences) was added to the sample to a final concentration of 0.5%. Samples were allowed to
rehydrate the strips overnight and focusing was performed on an IPGphor IEF system (Amersham Biosciences) for 4h (1h at 500V, 1h at 1000V, and 2h at 8000V). The buffer was then exchanged on the strips by soaking in 50mM Tris, pH 8.8, 6M urea, 30% glycerol, 2% SDS, 2 mg/mL DTT and a trace of bromophenol blue for 15min. Strips were layered horizontally on a 1mm thick 15% acrylamide SDS-gels with a 4% stacking gel for electrophoresis in the second dimension. Immunoblotting of the spots from the 2D gel was performed as described for regular immunoblots.

2.2-15: Statistics

Statistical analyses were carried out using Prism 4.0 software (GraphPad Software, San Diego, CA). When more than 2 groups are present, they were compared using one-way ANOVA with Newman-Keuls post-hoc analysis. For two groups, Student’s t-test is applied. p < 0.05 was considered statistically significant.
CHAPTER 3: Arp2/3- and cofilin-coordinated actin dynamics is required for insulin-mediated GLUT4 translocation to the surface of muscle cells

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GLUT4 vesicles are actively recruited to the muscle cell surface upon insulin stimulation. Key to this process is Rac-dependent reorganization of filamentous actin beneath the plasma membrane, but the underlying molecular mechanisms had yet to be elucidated. Using L6 rat skeletal myoblasts stably expressing myc-tagged GLUT4, we found that Arp2/3, acting downstream of Rac-1 GTPase, is responsible for the cortical actin polymerization evoked by insulin. siRNA-mediated silencing of either Arp3 or p34 subunits of the Arp2/3 complex abrogated actin remodelling and impaired GLUT4 translocation. Insulin also led to dephosphorylation of the actin-severing protein cofilin on Ser-3, mediated by the phosphatase slingshot. Cofilin dephosphorylation was prevented by strategies depolymerizing remodelled actin (latrunculin B or p34 silencing), suggesting that accumulation of polymerized actin drives severing to enact a dynamic actin cycling. Cofilin knockdown via siRNA caused overwhelming actin polymerization that subsequently inhibited GLUT4 translocation. This inhibition was relieved by re-expressing *Xenopus* wild type cofilin-GFP but not the S3E-cofilin-GFP mutant that emulates permanent phosphorylation. Transferrin recycling was not affected by depleting Arp2/3 or cofilin. These results suggest that cofilin dephosphorylation is required for GLUT4 translocation. We propose that Arp2/3 and cofilin coordinate a dynamic cycle of actin branching and severing at the cell cortex, essential for insulin-mediated GLUT4 translocation in muscle cells.
3.2: Introduction

A major function of insulin is to regulate glucose uptake by muscle and fat tissues. This is achieved through a rapid and dynamic gain in GLUT4 at the cell surface (131,221,222). Notably, this process becomes defective in insulin resistance states and type 2 diabetes (223-226). To date, defects in insulin signalling and GLUT4 traffic per se have been invoked to underlie such defects (227,228).

Upon normal insulin action, the recruitment of IRS-1/2 to active insulin receptors leads to activation of PI3K (146,229). In muscle cells, signalling bifurcates downstream of PI3K into two independent arms characterized by phosphorylation of Akt (147) and GTP activation of the small GTPase Rac-1 leading to actin remodelling (161,175). The two pathways are independent of one another because neither Akt dominant-negative mutants (147) nor the Akt inhibitor (182) prevent insulin-induced Rac activation or its consequent actin remodelling, and disruption of Rac-1 via siRNA fails to reduce Akt phosphorylation by insulin (177). Both signalling arms are required to elicit proper insulin-mediated GLUT4 translocation as perturbation of either one significantly reduces the GLUT4 response to insulin in muscle cells (131,147,157,177).

While much emphasis has been placed on the effectors downstream of Akt such as AS160 and Rab GTPases (154,155,158,230-232), the function of Rac-1 remains less explored. Rac-1 belongs to the small Rho GTPase family whose activity is regulated by GTP loading (180,233). Insulin promotes GTP loading of Rac-1 within the first 1-5 minutes of stimulation (175,176). Once activated, Rac-1 induces the reorganization of cortical actin filaments (161,177). This actin remodelling is a critical component in insulin-stimulated GLUT4 translocation because overexpression of a dominant negative Rac mutant (161) or siRNA-mediated Rac-1 knockdown (177) not only prevent actin
remodelling but also markedly diminish the insulin-mediated recruitment of GLUT4 to the surface. A similar reduction in insulin response is observed upon preventing actin remodelling with inhibitors of actin polymerization such as LB and CD (160,161), or by precluding actin depolymerization with jasplakinolide (165). Altogether, these findings reveal the importance of peripheral actin reorganization in insulin-dependent GLUT4 translocation in muscle cells. However, the precise regulation of this dynamic actin change, and the elements acting downstream of Rac-1 are undefined. Moreover, a model that incorporates both actin polymerization and depolymerization, as required for dynamic remodelling, has not been proposed.

Here we test the hypothesis that insulin produces a dynamic regulation of actin remodelling involving cycles of branching and depolymerization. Using a well-established muscle cell model of L6 GLUT4myc myoblasts displaying insulin-induced GLUT4myc exocytosis, we identify the Arp2/3 complex as a downstream effector of Rac-1 that governs cortical actin polymerization, and cofilin as a regulator promoting actin filament depolymerization. Cofilin dephosphorylation requires prior buildup of polymerized actin driven by Arp2/3. Therefore, an integrated mode for active actin cycling is proposed that enables insulin-mediated GLUT4 translocation/insertion into the muscle cell membrane.
3.3: Results

3.3-1: Arp2/3 is required for insulin-mediated actin remodelling

We have previously reported that insulin causes marked actin polymerization at the cortical periphery of L6 myoblasts and myotubes, that has a branched appearance when visualized by fluorescence (161,162) and electron (187) microscopy, and that manifests even in suspended myoblasts lacking stress fibers (175,187). The small GTPase Rac is a major regulator of such cortical actin remodelling (161,175,177). Known effectors of the Rho-family of small G proteins that modify actin filament organization in general include the branching complex Arp2/3 (234), capping proteins such as gelsolin and severing proteins such as cofilin (67). Here we hypothesize that Arp2/3 acts directly downstream of Rac-1 to initiate the insulin-dependent actin polymerization process. Arp2/3 is a seven-subunit complex consisting of Arp2, Arp3, p16, p20, p21, p34, and p40 (234). By transiently expressing the Arp3-GFP subunit into L6 GLUT4myc myoblasts, we sought to examine changes in the localization of Arp2/3 following insulin stimulation. In the unstimulated state, myoblasts displayed normal cellular stress fibers and Arp3-GFP was mainly cytosolic (Figure 3-1). When challenged with insulin, an actin meshwork formed at the periphery of the cells and Arp3-GFP redistributed to the zone of remodelled actin, suggesting its involvement at this region (Figure 3-1).
Figure 3-1: Arp3-GFP colocalizes with remodelled actin following insulin stimulation in myoblasts. L6 GLUT4myc myoblasts were transiently transfected with Arp3-GFP. Following 3 h serum starvation, cells were stimulated with 100 nM insulin for 10 min. Subsequent staining of F-actin by rhodamine-phalloidin was performed to observe the changes in the localization of Arp3-GFP with respect to the remodelled actin. Representative images of 4 independent experiments are shown. Bars, 20 µm.

To functionally establish the role of Arp2/3 in insulin-responsive actin remodelling, we interfered with the complex by silencing expression of its Arp3 subunit via siRNA-mediated knockdown. Significant down-regulation of Arp3 (by 77%) was achieved in siArp3-treated cells compared to cells treated with a non-related siRNA (siNR) sequence (Figure 3-2A). In Arp3 knockdown cells, the aspect and density of basal-state stress fibers remained unchanged. However, the insulin-induced actin remodelling was lost (Figure 3-2B). Quantitative analysis of remodelled actin revealed a 70% decrease in dorsal actin remodelling upon Arp3 down-regulation compared to control NR-treated cells (Figure 3-2C).
Figure 3-2: Down-regulation of Arp3 prevents the formation of remodelled actin and reduces GLUT4 translocation following insulin stimulation in myoblasts. A) Myoblasts were transfected with 200 nM of non-related (NR) siRNA control or Arp3 siRNA for 72 h. Total cell lysates were prepared and 10 µg protein were loaded and immunoblotted for Arp3 and actinin-1 (as loading control). Representative blots of 5 independent experiments are shown. B) Myoblasts transfected with NR or Arp3 siRNA were treated with/without insulin for 10 min followed by staining surface GLUT4myc in non-permeabilized cells, then permeabilized to label actin filaments with rhodamine-phalloidin. Dorsal actin remodelling was calculated from the pixel quantification in fluorescence optical cuts of the dorsal surface of adhered myoblasts (see Methods). Representative images of 3 independent experiments are shown. Bars, 20 µm. C) Quantification of changes in insulin-stimulated dorsal actin remodelling relative to NR control (mean ± S.E.). D) Quantification of fold increases in surface GLUT4myc relative to NR basal in NR and Arp3 knockdown conditions (mean ± S.E., #p<0.05).

This deleterious effect was further validated by inhibiting the function of Arp2/3 via down-regulation of another Arp2/3 subunit, p34. As with siArp3, siRNA-mediated knockdown of p34 (sip34) by 60% also prevented the formation of remodelled actin structures at the cell periphery upon insulin stimulation (Figure 3-3A,B).
Figure 3-3: Down-regulation of p34 inhibits the formation of remodelled actin and decreases GLUT4 translocation. A) L6GLUT4myc myoblasts were transfected with 200 nM of non-related (NR) siRNA control or p34 siRNA for 72 h. Total cell lysates were prepared and 10 µg protein were loaded and immunoblotted for p34 and actinin-1 (as loading control). Representative blots of 5 independent experiments are shown. B) Myoblasts transfected with NR or p34 siRNA were treated with/without insulin for 10 min followed by co-staining of surface GLUT4myc in the non-permeabilized state, then permeabilized to label actin filaments with rhodamine-phalloidin. Representative images of 5 independent experiments are shown. Bars, 20 µm. C) Quantification of changes in insulin-stimulated dorsal actin remodelling relative to NR control (mean ± S.E.). D) Quantification of fold increases in surface GLUT4myc relative to NR basal in NR and p34 knockdown conditions (mean ± S.E., #p<0.05).

To illustrate that Arp2/3 functions downstream of Rac-1 to generate the dorsal actin meshwork, we took advantage of a constitutively active Rac-GFP mutant (GFP-CA-Rac) that remolds actin without insulin stimulation when transfected into myoblasts. In the control of siNR-transfected cells expressing GFP-CA-Rac, distinctive actin remodelling was observed, compared to neighboring non-transfected cells.
However, GFP-CA-Rac failed to elicit actin remodelling after Arp3 knockdown (Figure 3-4). This result suggests that Arp2/3 is a major effector downstream of Rac governing actin remodelling in muscle cells.

**Figure 3-4: Arp2/3 functions downstream of Rac in insulin signal pathway.** L6GLUT4myc myoblasts transiently expressing GFP-CA-Rac were transfected with NR or Arp3 siRNA. Following 3 h serum starvation, unstimulated cells were labelled with rhodamine-phalloidin. Representative images of 3 independent experiments are shown. Bars, 20 µm.

3.3-2: Depletion of Arp2/3 reduces insulin-mediated GLUT4 gain on the cell surface

Because inhibition of Arp2/3 function averted actin remodelling, we explored its effect on insulin-responsive GLUT4 translocation. Following siRNA-mediated disruption of Arp2/3 function, co-staining of surface GLUT4myc and intracellular F-actin was applied to identify cells lacking the insulin-induced actin rearrangement and their corresponding GLUT4myc levels on the plasma membrane. Surface GLUT4myc showed the typical speckled distribution (162,230); that may suggest insertion into hot-spots. These may represent specific plasma membrane domains or may correspond to ruffled areas supported by the remodelled actin, consistent with the predominant localization of GLUT4myc in ruffles determined by immuno-gold labeling and scanning electron microscopy (165). Down-regulation of Arp3 did not alter the surface level of
GLUT4myc in the basal state, compared to control cells treated with siNR (Figure 3-2B). In contrast, siRNA to Arp3 not only prevented insulin-mediated peripheral actin remodelling but also significantly decreased the amount of GLUT4myc at the plasma membrane (Figure 3-2B). Quantification of surface GLUT4myc indicated a 44% inhibition in GLUT4 translocation after the down-regulation of Arp3 (Figure 3-2D). Furthermore, a strong reduction (60%) was achieved by knocking down the p34 subunit of Arp2/3 (Figure 3-3B,D). Neither Arp3 nor p34 knockdown altered the overall insulin-stimulated Akt phosphorylation (Figure 3-5), suggesting that the Akt signalling arm of insulin action remained intact.

**Figure 3-5: Down-regulation of Arp3 or p34 does not interfere with insulin-stimulated Akt phosphorylation.** L6GLUT4myc myoblasts were transfected with NR, p34, or Arp3 siRNA. Insulin (100 nM) was applied for 10 min after 3 h of serum starvation. Total cell lysates were then collected and immunoblotted for A) p34, P-Akt, actinin-1 and B) Arp3, P-Akt, actinin-1. Representative blots of 3 independent experiments are shown.

To demonstrate the specificity of Arp2/3 in GLUT4 translocation, we rescued Arp2/3 expression by transfecting *Dictyostelium discoideum* Arp3-GFP, which is resistant to siArp3 due to variance within the targeted sequence, into siArp3-treated cells. Under this setting, insulin-stimulated actin remodelling was restored (Figure 3-6A,B), and the deleterious effect of Arp3 down-regulation on insulin-mediated GLUT4 translocation was alleviated (Figure 3-6C). More importantly, expression of
Dictyostelium Arp3-GFP alone did not change the basal actin filament morphology or surface GLUT4 in unstimulated cells, indicating that Arp2/3 only exerts its functional action upon insulin stimulation.

**Figure 3-6:** Expression of *Dictyostellium* Arp3-GFP restores actin remodelling and GLUT4 translocation in Arp3 knockdown myoblasts. A) *Dictyostellium* Arp3-GFP was transiently expressed in L6GLUT4myc myoblasts transfected with NR or Arp3 siRNA. After 3 h serum starvation, cells were challenged with 100 nM insulin for 10 min. F-actin was stained with rhodamine phalloidin to indicate actin remodelling. Representative images of 4 independent experiments are shown. Bars, 20 µm. B) Quantification of changes in insulin-stimulated dorsal actin remodelling relative to siNR control (mean ± S.E.). C) Myoblasts with NR or Arp3 siRNA were transfected with/without *Dictyostellium* Arp3-GFP. Following serum depletion, cells were stimulated with insulin for 10 min. Surface GLUT4myc content was measured via single cell
detection of surface myc fluorescent intensity and quantified as fold increases over siNR basal (mean ± S.E., n=6, *p<0.05).

The fact that depletion of Arp2/3 components only altered the insulin-dependent component of surface GLUT4 but did not change the basal levels of the transporters at the cell membrane suggests that the constitutive recycling of GLUT4 does not require Arp2/3 input. To establish if the effect of Arp2/3 interference is selective to GLUT4 traffic, we examined the effect of depletion of p34 of the Arp2/3 complex on transferrin recycling, which depends on endosome recycling. As shown in Figure 3-7, transferrin recycling in either basal or insulin stimulated state was similar in cells treated with siRNA to p34 as in siNR-treated cells, implying that the inhibition of Arp2/3 did not affect the traffic of transferrin.

Figure 3-7: Knockdown of either p34 or cofilin does not alter transferrin recycling. L6GLUT4myc myoblasts were treated with NR, p34, or cofilin siRNA. Following 3 h serum starvation, to $^{125}$I-Tf recycling was determined as described in Materials and Methods, in basal and insulin stimulated conditions. Transferrin recycling is displayed as the ratio of externalized Tf over internalized Tf (mean± S.D.). n=3.

The above results indicate that Arp2/3 is required for cortical actin remodelling and GLUT4 translocation including full exposure at the cell surface. To ascertain that the
effect of Arp2/3 knockdown is independent of possible changes in actin stress fibers, we analyzed suspended myoblasts devoid of stress fibers. In this rounded-up configuration, myoblasts show actin filaments exclusively at the cell periphery (175,187,188). Myoblasts treated with siRNA to p34 of the Arp2/3 complex or with siNR were suspended, stimulated with insulin and analyzed for actin remodelling and surface GLUT4 levels. Whereas siNR-treated cells showed the habitual cortical actin arborizations and gain in surface GLUT4 in response to insulin, sip34 treatment markedly abolished both responses to insulin (Figure 3-8). These results suggest that it is the cortical actin structures regulated by Arp2/3 that are required for GLUT4 translocation in response to the hormone.

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**Figure 3-8: Stress fiber does not contribute to the decrease in GLUT4 traffic observed in p34 knockdown.** p34 knockdown abolishes cortical actin remodeling in rounded-up myoblasts and prevents gain in surface GLUT4myc in response to insulin. Bars, 10 µm. Representative of 2 independent experiments.

3.3-3: Insulin causes cofilin dephosphorylation that depends on slingshot

Actin dynamics is regulated by concerted actin polymerization and depolymerization. In fact, a spatial/temporal burst in actin polymerization and branching, as caused by Arp2/3, requires available sources of actin monomer, which are typically
provided by the continuous depolymerization of actin filaments (235,236). Hence, we sought to identify molecules in the actin-depolymerization pathway that may respond to insulin, which would contribute to the balance of actin dynamics. ADF/cofilin are highly related, actin-severing and monomer-sequestering proteins, whose activity is mainly regulated by the phosphorylation status of Ser3 (74). Phosphorylation and dephosphorylation of ADF/cofilin leads to its inactivation and activation, respectively. Insulin elicited ADF/cofilin dephosphorylation in cells that do not represent metabolically-determining tissues and do not express GLUT4 (HT4 neuronal and 293T cells) (213,237), but the function of cofilin in these cells and the metabolic consequences of its activation were not investigated. We therefore examined whether insulin stimulation in muscle cells alters the ADF/cofilin phosphorylation status and its possible contribution to GLUT4 traffic. We utilized a rabbit antibody that recognizes both ADF and cofilin-1 and their phosphorylated forms to an equal extent and cofilin-2 to a somewhat lesser extent (214). By two-dimensional gel electrophoresis of L6 cell lysates, we calculated the relative ratio of cofilin:ADF in L6 myoblasts to be 7:1. This approach also revealed that about 9% of the cellular content of cofilin is phosphorylated (inactive) in unstimulated cells, and insulin caused a 4-fold decrease in the phosphorylation of this protein (Figure 3-9).
Figure 3-9: Relative percent of cofilin, ADF, phosphorylated-cofilin and phosphorylated ADF. Basal and insulin-stimulated L6GLUT4myc myoblasts were lysed and subjected to two-dimensional gel electrophoresis and assessment of ratios of ADF, cofilin and their phosphorylated versions, essentially as described in methods.

By SDS-PAGE and immunoblotting with an antibody selective to cofilin phosphorylated on P-Ser3, phosphorylation of cofilin in unstimulated cells was also ascertained (Figure 3-10A). Upon insulin stimulation, a reduction in the level of phosphorylated cofilin (P-cofilin) was observed without changes in total cofilin, indicating a shift to its active state (Figure 3-10A). This was evident as early as 3 min and was most significant 10 min following insulin treatment (Figure 3-10B). Inhibiting PI3K with wortmannin prevented cofilin dephosphorylation in response to insulin (not shown), paralleling the response in 293T cells (237).
**Figure 3-10: Insulin stimulation in L6GLUT4myc muscle cells causes dephosphorylation of cofilin.** L6GLUT4myc myoblasts were serum-starved for 3 h prior to insulin stimulation (100 nM) for the indicated time periods. A) Total lysates immunoblotted for P-cofilin, cofilin, P-Akt, actin (as loading control). Representative blot of 4. B) Quantification of insulin-dependent cofilin dephosphorylation expressed as P-Cofilin/Cofilin ratio relative to time 0 (n=4, mean ± S.E., #p<0.05).

The balance of cofilin phosphorylation/dephosphorylation is primarily achieved by the action of kinases LIMK and testicular kinase, and of the phosphatases slingshot (SSH), chronophin (238) and to some degree PP1/PP2A (213). Because net dephosphorylation of cofilin was observed following insulin stimulation, we examined first the participation of the phosphatases in this response. Treatment of myoblasts with SSH1 siRNA reduced the expression of SSH1 by 73% (Figure 3-11A). Down-regulation of this phosphatase notably prevented the dephosphorylation of cofilin evoked by insulin compared to the degree observed in NR siRNA controls (Figure 3-11B). This revealed that insulin signals primarily via SSH1 to dephosphorylate cofilin, which would lead to cofilin activation.

**Figure 3-11: Insulin-induced dephosphorylation of cofilin is slingshot-dependent.** A-B) Lysates from myoblasts treated with SSH1 siRNA (siSSH1) were prepared to determine the knockdown effect on SSH1 and its contribution towards insulin-induced cofilin dephosphorylation by immunoblotting for P-Cofilin.

Regarding the kinases mediating cofilin phosphorylation, LIMK is attractive since
it is typically activated by phosphorylation via Rac-dependent, p21-activated-kinase (80). Although LIMK1 knockdown did not increase the steady-state phosphorylation of cofilin in the basal state, LIMK knockdown potentiated cofilin dephosphorylation in response to insulin (Figure 3-12). This observation suggests that, in response to insulin, LIMK1 may partially restore phosphorylation of cofilin, which is however more dominantly dephosphorylated by SSH1.

![Image](3-12.png)

**Figure 3-12: Down-regulation of LIMK1 does not affect insulin-induced cofilin dephosphorylation.** Lysates from myoblasts treated with LIMK1 siRNA were prepared to determine the extent of LIMK knockdown and its contribution towards insulin-induced cofilin dephosphorylation by immunoblotting for P-Cofilin. Representative blots of 3 independent experiments are shown.

3.3-4: Arp2/3-mediated actin remodelling signals to insulin-stimulated cofilin dephosphorylation

The net effect of cofilin dephosphorylation induced by insulin suggests the activity of SSH1 outweighed that of LIMK1. One of the possible explanations could be a surge in the phosphatase activity of SSH1 following insulin stimulation. Indeed, addition of F-actin to purified SSH1 markedly augments its phosphatase activity (239,240). Analogously, the remodelled actin produced in muscle cells by insulin could serve as the stimulus that boosts the activity of SSH1 *in vivo* to cause a shift towards cofilin dephosphorylation because SSH1 accumulated at the remodelled actin following insulin
stimulation (Figure 3-13). This shift in localization to the peripheral remodelled actin is insulin-dependent as SSH1 in unstimulated cells was uniformly distributed in the cytosol (results not shown).

Figure 3-13: SSH1 redistributes to the zone of actin remodelling upon insulin stimulation. L6 myoblasts were transiently transfected with SSH1 cDNA, serum-deprived cells and stimulated with 100 nM insulin for 10 min, followed by fixation and permeabilization, to allow detection of transfected SSH1 and F-actin. Representative images of 3 independent experiments are shown. Bars, 10 µm.

Knowing that the inhibition of Arp2/3 prevented actin remodelling, we examined this hypothesis by examining changes in P-cofilin levels upon p34 knockdown. In control cells treated with siNR, cofilin dephosphorylation occurred normally as evident from the decrease in P-cofilin level after 5 and 10 min of insulin stimulation (Figure 3-14A). In contrast, and notably, this insulin-dependent decrease in the level of P-cofilin was lacking in p34 knockdown cells (Figure 3-14A). Similar results were obtained when the actin filament-disrupting agent LB was used to stop the formation of remodelled actin by insulin. Under LB treatment, the dephosphorylation of cofilin by insulin was also prevented, so that the levels of P-cofilin at 5 and 10 min of stimulation were comparable to that of unstimulated cells (Figure 3-14B). In neither case was the ability of insulin to signal to Akt affected. These findings support the idea that the remodelled actin functions as a stimulus that augments the activity of SSH1 to generate the net
dephosphorylation of cofilin following insulin stimulation in muscle cells.

### Figure 3-14: Insulin-stimulated dephosphorylation of cofilin is dependent on remodelled actin. Myoblasts were: A) transfected with p34 siRNA or B) subjected to 250 nM latrunculin B treatment for 30 min, and the effect of insulin on P-Cofilin was assessed. Representative blots of >3 independent experiments are shown.

3.3-5: Cofilin knockdown promotes F-actin accumulation and decreases insulin-mediated GLUT4 translocation

Given that insulin activates cofilin via its dephosphorylation, it became intriguing to explore the functional implication that cofilin has on the dynamics of insulin-responsive actin remodelling. Immunofluorescence detection of endogenous cofilin in myoblasts revealed its redistribution from the cytosol to the peripheral zone where actin remodels upon insulin stimulation (Figure 3-15A). The localization of the phosphorylated form was determined using an antibody to phosphorylated ADF/cofilin that is compatible with immunofluorescence approaches. Co-staining of total cofilin and phosphorylated ADF/cofilin (P-AC) revealed that insulin stimulation decreased the ratio of P-AC/cofilin at the cell periphery/remodelled actin (by 31%, p<0.05), indicating that cofilin undergoes dephosphorylation in response to the hormone at this region of the cell (Figure 3-15B,C), consistent with the localization of SSH1.
Figure 3-15: Cofilin is localized to the remodelled actin upon insulin stimulation. A) L6GLUT4myc myoblasts were serum-starved for 3 h and stimulated with 100 nM insulin for 10 min, followed by labeling with rhodamine-phalloidin for F-actin and cofilin specific antibody for endogenous cofilin. Representative images of 3 independent experiments are shown. Bars, 10 µm. B) Serum-starved L6 myoblasts were stimulated with or without 100 nM insulin for 10 min, followed by fixation and permeabilization. The cells were labelled for phosphorylated AC and total cofilin using specific affinity-purified antibodies. C) Areas of similar size of the cell periphery containing remodelled actin, and background areas that did not contain any cells, were selected. The fluorescence intensity in these regions from 50 basal-state and 43 insulin-stimulated cells were determined, averaged and plotted as ratio of P-AC/cofilin signal (mean ± S.E., #p<0.05).

To explore if cofilin dephosphorylation is required for insulin action on actin dynamics and GLUT4 translocation, siRNA against cofilin was applied to achieve an 82% knockdown compared to the siNR control (Figure 3-16A). As expected from existing literatures (241, 242), down-regulation of cofilin caused a massive increase in the level of random F-actin aggregates in the basal state and this morphological change was scored via polymerized dorsal actin assay (Figure 3-16B,C). When challenged with insulin, these cells displayed a further elevation in polymerized actin (Figure 3-16C),

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suggesting that potentially there were still some available actin monomers to respond to the actin polymerization cues evoked by insulin. However, this remodelled actin was morphologically more diverse and was not confined to the cell periphery compared to that in NR control cells stimulated by insulin (Figure 3-16B).

Figure 3-16: Down-regulation of cofilin increases F-actin aggregates and reduces insulin-induced GLUT4 translocation. A) Total lysates from myoblasts transfected with NR or cofilin siRNA were prepared and immunoblotted for cofilin and actinin-1 (loading control). Representative blots of 5 independent experiments are shown. B) Myoblasts with siNR or siCofilin were treated with/without insulin followed by co-staining of surface GLUT4myc and F-actin. Representative images of 4 independent experiments are shown. Bars, 20 µm. C) Quantification of dorsal polymerized actin relative to NR basal after cofilin knockdown (mean ± S.E.). D) Quantification of fold increases in surface GLUT4myc relative to NR basal in NR and siCofilin conditions (mean ± S.E., #p<0.05).
Although insulin-dependent actin polymerization continued upon down-regulation of coflin, the visually excessive/abnormal remodelling suggests that the dynamics of F-actin at the zone of remodelling may have been impeded. We therefore explored whether under these conditions the insulin-dependent GLUT4 translocation was affected. As shown in Figure 3-16 B and D, coflin knockdown caused a major reduction (66%) in insulin-dependent gain in surface GLUT4, yet transferrin recycling, in either absence or presence of insulin, was not significantly disrupted (Figure 3-7). This observation allowed us to test whether coflin re-expression restores normal GLUT4 translocation, and if such restoration would be dependent on the phosphorylation status of coflin. Transfecting *Xenopus* coflin-WT-GFP into myoblasts with down-regulated coflin expression restored the normal F-actin pattern in the basal state by eliminating the excessive F-actin accumulation (Figure 3-17A,B). Moreover, such re-expression also alleviated the defect in insulin-dependent GLUT4 translocation (Figure 3-17C).

Strikingly, neither the recovery of actin dynamics nor that of GLUT4 translocation was achieved when the inactive coflin-S3E-GFP mutant was transfected into myoblasts with down-regulated coflin. This mutant is unable to severe actin filaments. Overall, these results argue that the insulin-induced increase in the severing function of coflin is critical for the dynamics of actin remodelling, which in turn allows proper insulin-stimulated GLUT4 translocation to proceed.
Figure 3-17: Expression of *Xenopus* cofilin-WT-GFP, but not cofilin-S3E-GFP mutant, restores normal F-actin morphology and GLUT4 translocation. A) L6GLUT4myc myoblasts transfected with NR or cofilin siRNA were further transfected with cDNA to either GFP as control, cofilin-WT-GFP, or cofilin-S3E-GFP. Following serum starvation, F-actin was labelled with rhodamine-phalloidin in the basal state to detect changes in actin morphology. Representative images of 6 independent experiments are shown. Bars, 20 µm. B) Quantification of F-actin aggregates relative to NR+GFP basal (mean ± S.E.). C) Surface GLUT4myc levels in siCofilin-treated myoblasts cotransfected with cofilin-WT or S3E mutant expression in siCofilin myoblasts were measured by fluorescent detection of single cells and are presented as fold increases in surface GLUT4myc relative to NR basal are also shown (mean ± S.E., n=6, #p<0.05).
3.4: Discussion

Since muscle is the major insulin-regulated storage of blood glucose, it is imperative to define the signalling events that promote the gain of GLUT4 at the surface to increase glucose uptake into this tissue. In parallel to the well-established Akt-AS160 pathway (154,230), we recently showed that insulin induces a rapid activation of the small GTPase Rac-1 that is PI3K-dependent and leads to cortical actin remodelling (161,165,175,177). Interestingly, Rac-1 activation and actin remodelling fail in several models of insulin resistance, such as elevated ROS and ceramide accumulation, despite intact upstream IRS-1 phosphorylation and PI3K activation, which reinforces the significance of the Rac-1 pathway in insulin-stimulated GLUT4 translocation (165,177,243). Here we identify Arp2/3 as the effector downstream of Rac-1 that promotes formation of remodelled actin, while the activity of cofilin maintains the active turnover of actin in the remodelling zone. Disruption of either protein yielded abnormal actin remodelling and subsequent inhibition in insulin-mediated GLUT4 translocation (Figure 3-2, 3-3, 3-12).

3.4-1: Insulin induces concerted actin branching and severing

F-actin contributes to maintaining structural integrity, promoting cellular migration, and aiding in vesicle traffic. In order to be functional, these cellular processes depend on the dynamic nature of actin regulation. Cytoplasmic actin exists as monomers and oligomerized F-actin. Uncapped F-actin undergoes constant polymerization at the barbed ends while depolymerization occurs at the point ends to regenerate a steady pool of monomeric actin for further rounds of polymerization (236). Such dynamic turnover is tightly controlled by actin-modifying proteins including actin nucleating and severing proteins (236). Arp2/3 initiates branching at 70° on existing actin filaments and
is firmly established in the Rac-dependent formation of lamellipodia in migrating cells (47,244). Here we report that Arp2/3 contributes to the Rac-mediated, cortical actin remodelling following insulin stimulation. Down-regulation of Arp2/3 through siRNA against its Arp3 or p34 subunits abrogated insulin-induced actin remodelling, suggesting that Arp2/3-initiated actin polymerization is responsible for the genesis of dorsally remodelled actin in muscle cells (Figure 3-2B, 3-3B). Overexpressing Arp3-GFP did not promote actin polymerization in the basal state (Figure 3-1), suggesting that Arp2/3 activation is tightly controlled by insulin stimulation. Conceivably, this occurs in response to insulin-activated Rac-1 and its downstream effectors. Indeed, constitutively active Rac caused Arp2/3-mediated actin branching (Figure 3-4).

In both L6 muscle cells (this study) and 3T3-L1 adipocytes (245), insulin caused Arp2/3 colocalization with the peripheral remodelled actin, and cortical actin remodelling is required for GLUT4 translocation (164-166). However, this phenomenon is differentially regulated in both cell types, as only in the muscle cells is the insulin-dependent actin remodelling downstream of PI3K (162,175). Moreover, the Rho-family G proteins engaged by each cell type also differ, Rac-1 determining actin remodelling in the muscle cells and TC10 (related to Cdc42) in adipocytes (169,175). Arp2/3 is regulated by the nucleation promoting factors N-WASP, WAVE and cortactin (246-248), and a dominant negative mutant of N-WASP prevented the TC10-mediated insulin-induced actin reorganization in adipocytes (245). In contrast, WAVE, rather than N-WASP, is the nucleating factor downstream of Rac-1 that activates Arp2/3 and its consequent actin filament branching (247). Thus, WAVE2 may be the Arp2/3 activator in insulin-stimulated muscle cells, a possibility worthy of future exploration.
Insulin also activated the actin severing protein cofilin by inducing its dephosphorylation on Ser3 (Figure 3-10). This may be required to generate a dynamic, rapid turnover of the branching network, and to provide a continuous supply of monomeric actin. Because cofilin phosphorylation and dephosphorylation are governed by LIMK1 and SSH1 respectively (75,77), we propose that insulin must activate the phosphatase and this enzyme has a predominant effect over the kinase to produce the net dephosphorylation (249). In muscle cells, cofilin is already phosphorylated in the absence of stimuli (Figure 3-10), ostensibly due to a low, tonic activity of LIMK1 and a relatively inactive SSH1. Upon insulin stimulation, cofilin undergoes SSH1-dependent dephosphorylation (Figure 3-11). The surge in SSH1 activity may arise in response to the formation of the cortical network of branched, polymerized actin, since F-actin binding is required for significant phosphatase activity of SSH1 (239,240,249). This model is supported by our observations that LB and p34 knockdown, strategies that prevent insulin-induced actin polymerization, avert cofilin dephosphorylation (Figure 3-14). Consistent with this scenario, SSH1 concentrated along the peripherally remodelled actin upon insulin stimulation (Figure 3-13). Moreover, there is precedent for F-actin tilting the balance from LIMK1 to SSH1 dominance over cofilin (250). A dynamic interplay between LIMK1 and SSH1 likely enables spatial temporal control over cofilin activity especially at the zone of insulin-induced actin remodelling.

3.4-2: Arp2/3 activation and cofilin dephosphorylation are required for insulin-dependent GLUT4 translocation

A key observation of this study is that silencing two components of the Arp2/3 complex prevents GLUT4 translocation in response to insulin. This result is reminiscent of the well-documented abrogation of GLUT4 translocation by LB in fat cells (163,164)
and muscle cells (161,166) in culture, and in primary adipocytes (168) and muscle tissue (167). However, since LB inhibits actin remodelling by sequestering actin monomers, it impedes actin polymerization not only at the cell cortex but also at the level of stress fiber (251). Instead, disruption of Arp2/3 allowed us to assign the selective participation of branched actin remodelling in GLUT4 translocation, irrespective of stress fibers or other actin filament-containing structures.

When actin severing was impaired by siRNA-mediated knockdown of coflin, actin polymerization was extensive and widely prevalent across the cell (Figure 3-16B), and this abnormality paralleled a reduction in insulin-dependent GLUT4 translocation (Figure 3-16D). This finding is reminiscent of the effect of jasplakinolide, an actin-stabilizing agent that causes excessive actin aggregates resembling those produced in cells with coflin knockdown (252). Likewise, jasplakinolide also interferes with GLUT4 vesicle traffic to the surface of muscle and adipose cells (164-166). All these considerations point to the need for a dynamic, concerted actin polymerization and severing induced by insulin and required for GLUT4 translocation. Perhaps the strongest support of this model is the fact that the defect in GLUT4 traffic caused by coflin knockdown was rescued by expression of active coflin-WT-GFP (which is amenable to phosphorylation and dephosphorylation) but could not be restored by the expression of inactive coflin-S3E-GFP (Figure 3-17C). These results buttress the participation of insulin-induced, coflin-mediated actin depolymerization to facilitate GLUT4 translocation, and show for the first time that coflin dephosphorylation is essential for insulin-dependent vesicle traffic.

3.4-3: Possible mechanisms whereby a dynamic actin network supports GLUT4 traffic

The remodelled actin filaments may act as a tether for GLUT4 vesicles close to the
plasma membrane, so that their subsequent docking/fusion can occur more readily (161,165,187). Indeed, in insulin-stimulated muscle cells, GLUT4 vesicles accumulate within the cortical actin mesh visualized by electron microscopy (165,187). GLUT4 itself may tether to actin filaments via α-actinin-4 (188,189), and indeed upon α-actinin-4 silencing, GLUT4 vesicles do not accumulate at the muscle cell periphery and there is no insulin-dependent gain in surface GLUT4 (188). Similarly, when actin filament remodelling is inhibited by expressing dominant negative Rac or by LB, the enrichment of GLUT4 beneath the plasma membrane is lost, leading to the collapse of GLUT4 vesicles back to perinuclear regions (187). It is also plausible that the remodelled actin clusters insulin signalling molecules close to GLUT4 vesicles near the plasma membrane (162). Although preventing actin remodelling by Arp2/3 knockdown did not reduce the overall level of P-Akt (Figure 3-5), failure to accumulate phosphorylated Akt near the membrane may be a factor in the loss of insulin-induced GLUT4 translocation (253-256).

On the other hand, actin polymerization is abundant in cells with down-regulated cofilin yet the resulting polymerized actin filaments seem morphologically distinct and disseminated across the cell (Figure 3-16B). We speculate that, in this case, the impaired gain in surface GLUT4 is due to vesicle retention in a static mesh that no longer undergoes severing. By analogy, in neuronal cells, thick patches of submembrane F-actin present at rest act as a barrier to inhibit basal exocytosis of neurotransmitters (257). In cells with down-regulated cofilin, the imbalance in dynamic cycles of actin depolymerization and branching may lead to uncoordinated growth of ‘static’ actin filaments that could similarly turn into a barrier for GLUT4 vesicles,
preventing them from gaining access to interaction with membrane SNAREs required for fusion (187).

We propose a model (Figure 3-18) whereby insulin stimulation leads to Arp2/3 activation downstream of Rac that initiates dorsal actin polymerization. The increase in polymerized actin stimulates the phosphatase activity of SSH1, leading to coflin dephosphorylation and consequential increase in its severing action. Active actin depolymerization by coflin can then free up actin monomers for Arp2/3-dependent polymerization and a dynamic actin turnover of insulin-stimulated actin remodelling is achieved. This dynamic status must be sustained to allow GLUT4 vesicle positioning and proper interaction with elements of the vesicle fusion machinery. This framework will allow testing the fidelity of its individual steps during insulin resistance, and may reveal so far unsuspected steps that may be altered in this condition.

Figure 3-18. Proposed mechanism of insulin-regulated actin dynamics in muscle cells. Insulin stimulation in muscle cells promotes dynamic actin remodelling. The formation of the remodelled actin is achieved by the polymerization activity of Arp2/3 acting downstream of active Rac. The accumulation of polymerized F-actin poses a stimulatory factor in the phosphatase activity of SSH, which leads to net dephosphorylation and activation of coflin. Hereon, the actin severing function of coflin
maintains the flexibility of remodelled actin and enables regeneration of free monomeric actin for further polymerization. This active cycling of actin mediated by insulin keeps proper actin dynamics at the cortical zone in order to facilitate GLUT4 insertion onto the cellular surface.
CHAPTER 4: Rac-1 superactivation triggers insulin-independent GLUT4 translocation that bypasses signalling defects exerted by JNK- and ceramide-induced insulin resistance

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4.1: Abstract

Insulin activates a cascade of signalling molecules, including Rac-1, Akt, and AS160, to promote the net gain of GLUT4 at the plasma membrane of muscle cells. Interestingly, constitutively active Rac-1 expression results in a hormone-independent increase in surface GLUT4, yet the molecular mechanism and significance behind this effect remains unresolved. Using L6 myoblasts stably expressing myc-tagged GLUT4, we found that overexpression of constitutively active, but not wild type Rac-1, sufficed to drive GLUT4 translocation to the membrane, of comparable magnitude to that elicited by insulin. Stimulation of endogenous Rac-1 by Tiam1 overexpression elicited a similar hormone-independent gain in surface GLUT4. This effect on GLUT4 traffic could also be reproduced by acutely activating a Rac-1 construct via rapamycin-mediated heterodimerization. Strategies triggering Rac-1 'superactivation' (i.e., to levels above those attained by insulin alone) produced a modest gain in plasma membrane PI\textsubscript{(3,4,5)}P\textsubscript{3}, moderate Akt activation and substantial AS160 phosphorylation, which translated into GLUT4 translocation and negated the requirement for IRS-1. This unique signalling capacity exerted by Rac-1 superactivation bypassed the defects imposed by JNK- and ceramide-induced insulin resistance and allowed full and partial restoration of GLUT4 translocation response, respectively. We propose that potent elevation of Rac-1 activation alone suffices to drive insulin-independent GLUT4 translocation in muscle cells, and such strategy might be exploited to bypass signalling defects during insulin resistance.
4.2: Introduction

Insulin-stimulated glucose uptake into skeletal muscle is a major determinant of whole body glucose homeostasis, as muscle is the primary tissue responsible for the vast majority of dietary glucose disposition (258). Insulin binding to its receptor at the muscle cell surface initiates a complex signalling cascade leading to the net recruitment of GLUT4-containing vesicles from an intracellular compartment to the plasma membrane (142). Identifying the full complement of insulin-triggered signals regulating GLUT4 translocation in muscle could provide a valuable asset in overcoming defects that lead to insulin resistance and its consequent type 2 diabetes.

In L6 muscle cells in culture, the first post-receptor event leading to GLUT4 translocation is the selective tyrosine phosphorylation of IRS-1 (145) with consequent activation of PI3K to elevate membrane P(3,4,5)P_3 (162,210). Downstream of PI3K, insulin signalling in muscle cells bifurcates into two independent pathways, characterized, respectively, by activation of the serine/threonine kinase Akt and the Rho-family GTPase Rac-1 (147,175,177). Akt in turn phosphorylates the protein AS160 to inhibit its GAP activity towards Rab GTPases (154,230,259). This enables the activation (GTP-loading) of Rabs 8A and 13 to prevail and provide mobilization cues for GLUT4 vesicle delivery to the muscle cell plasma membrane (156,157). The other signalling arm involves PI3K-dependent Rac-1 activation (GTP-loading) that triggers a cycle of branching and severing of cortical actin filaments (260). The resulting cortical mesh of branched actin filaments may serve as a tether for both signalling molecules and GLUT4 vesicles (162,177,187,188,260). Activation of Rac-1 is thought to depend on either activation of dedicated GEFs and/or inhibition of dedicated GAPs (180,261).
In vivo, muscle Akt is readily activated in response to insulin, and Akt-2 gene knockout mice show reduced insulin-dependent glucose uptake into muscle (151). Similarly, recent studies show that insulin activates Rac-1 in mouse muscle, and mice with muscle-specific Rac-1 gene deletion have diminished insulin-dependent GLUT4 translocation and glucose uptake (178,179). The latter results parallel observations of abated GLUT4 translocation in L6 muscle cells in culture depleted of Rac-1 via siRNA-dependent gene silencing (177).

Importantly, both Akt and Rac-1 are required for proper insulin-induced GLUT4 translocation, as overexpressing dominant-negative mutants of each one or silencing their endogenous gene expression, impair the insulin response of GLUT4 without mutually inhibiting one another (147,161,177). In spite of this apparent independence of the Akt and Rac-1 signalling arms, overexpression of constitutively active Rac-1 in muscle cells increases surface GLUT4 (181), though the molecular underpinnings are unknown. Here, we build on that observation and unravel the signalling mechanism.

Using acute (rapamycin-inducible) and chronic modalities of Rac-1 superactivation (i.e., activation surpassing the activation levels caused by insulin), including activation of a Rac-specific GEF, Tiam1, we achieved an insulin-comparable GLUT4 translocation response without input from insulin. Unexpectedly, this hormone-independent Rac-1 activation generated a modest increase in membrane-associated PI_{3,4,5}P_{3} that in turn caused mild Akt phosphorylation. Downstream signalling was amplified, achieving substantial AS160 phosphorylation and GLUT4 translocation. Rac-1 superactivation bypassed the requirement for IRS-1 phosphorylation. Finally, we show that Rac-1 superactivation relieves the GLUT4 traffic defect imposed by two strategies that cause
insulin resistance: activation of the stress kinase JNK and ceramide exposure. Hence, Rac-1 is mechanistically identified as a viable target for the treatment of insulin resistance in a cellular model. By crossover activation of Akt, Rac-1 activation enacts the full complement of insulin-derived distal responses that mobilize GLUT4 in muscle cells, independently of IRS-1 participation.
4.3: Results

4.3-1: Rac-1 activation via overexpression of GFP-CA-Rac or Tiam1-GFP induces a gain in surface GLUT4 independent of insulin

We first verified that elevating Rac-1 activity would raise GLUT4 surface levels. L6 myoblasts stably expressing myc-tagged GLUT4 (L6-GLUT4myc) were transiently transfected with wild-type (WT)- or constitutively active (CA)-Rac-GFP. In control L6-GLUT4myc cells, GFP expression alone did not change the basal surface GLUT4 level while insulin stimulation enhanced it by 2.2-fold (Figure 4-1A,B). GFP-WT-Rac overexpression did not alter surface GLUT4 levels compared to GFP-expressing control cells (Figure 4-1A,B). However, when GFP-CA-Rac was introduced, surface GLUT4 was significantly higher compared to that in neighbouring untransfected cells (Figure 4-1A). The 2.4-fold increase in basal surface GLUT4 elicited by GFP-CA-Rac overexpression was similar to that caused by insulin stimulation in the GFP-expressing controls (Figure 4-1B). Addition of insulin to GFP-CA-Rac expressing cells did not cause a further enhancement in surface GLUT4 compared to the already augmented amount detected in the absence of insulin (Figure 4-1A,B).
Figure 4-1: Overexpression of GFP-CA-Rac stimulates GLUT4 translocation in muscle cells. A-B) GLUT4myc myoblasts were transfected with GFP, GFP-WT-Rac, or GFP-CA-Rac followed by stimulation with insulin for 10 min or left untreated to measure changes in surface GLUT4myc and quantified relative to GFP basal (mean ± SE, *p<0.05). Representative images of 3 independent experiments are shown. Bar = 10 µm.

This gain in surface GLUT4 was specific to active Rac-1 because neither the overexpression of WT-Cdc42 nor CA-Cdc42, a closely related Rho-GTPase, raised surface GLUT4 compared to the level observed with CA-Rac overexpression (Figure 4-2A-B). More importantly, transient overexpression of GFP-CA-Rac did not alter the total amount of cellular GLUT4myc compared to the GFP controls (Figure 4-2C), which eliminates the possibility of elevated GLUT4myc expression contributing to the heightened surface GLUT4 level observed in GFP-CA-Rac expressing cells.
Figure 4-2: Overexpression of WT- or CA-Cdc42 does not produce an insulin-like increase in surface GLUT4 and GFP-CA-Rac expression does not change total GLUT4myc levels. A-B) GLUT4myc myoblasts were transfected with CFP, CFP-WT-Cdc42, or CFP-CA-Cdc42 followed by measurement of basal surface GLUT4 and quantified relative to CFP-expressing, unstimulated controls (basal) (mean ± SE, *p<0.05). Representative images of 3 independent experiments are shown. Bar = 10 µm. C) Total GLUT4myc in cells expressing GFP or GFP-CA-Rac was measured as described, and quantified relative to the levels in GFP-expressing control cells (mean ± SE). (n=3).

To validate the effect of transiently transfected GFP-CA-Rac, we activated endogenous Rac-1 through overexpression of a Rac-specific GEF, Tiam1 (262). As shown in the literature (263), in the absence of growth factor stimulation, Tiam1-GFP overexpression generated F-actin rich ruffles that resembled those evoked by GFP-CA-Rac, suggesting that the endogenous Rac-1 was activated in Tiam1 overexpressing cells (Figure 4-3A). Surface GLUT4 levels were significantly higher in Tiam1 overexpressing cells compared to GFP-expressing or untransfected cells (Figure 4-3B). The net gain in GLUT4 at the plasma membrane was again similar to the increase induced by insulin in GFP-expressing controls (Figure 4-3C). These results mirrored the data obtained in CA-Rac expressing cells, reinforcing the observation that potent activation of Rac-1 alone exerts a full, insulin-like response on GLUT4 traffic to the plasma membrane.
Figure 4-3: Overexpression of Tiam1-GFP induced Rac-1 activation and GLUT4 translocation in muscle cells. A) Myoblasts transfected with GFP, GFP-CA-Rac or Tiam1-GFP were permeabilized and stained with rhodamine phalloidin to visualize changes in F-actin. B-C) GLUT4myc myoblasts expressing GFP or Tiam1-GFP were stimulated with insulin for 10 min or left untreated followed by staining for surface GLUT4myc and quantification relative to GFP basal (mean ± SE, *p<0.05). Representative images of 3 independent experiments are shown. Bar = 10 µm.

To compare the level of Rac-1 activation achieved by insulin stimulation and GFP-CA-Rac overexpression, we analyzed the amount of GTP-loaded Rac in WT- or GFP-CA-Rac expressing myoblasts stimulated with or without insulin. GFP-WT-Rac overexpressing cells exhibited an increase in Rac-1 activation following 10 min of insulin stimulation (Figure 4-4). However, the level of GTP-loaded Rac-1 evoked by insulin was much lower compared to that in myoblasts overexpressing GFP-CA-Rac. Therefore, the effect achieved by GFP-CA-Rac expression in myoblasts can comparatively be
considered to be a Rac-1 superactivation, and this term is adopted throughout this manuscript.

Figure 4-4. Insulin stimulated Rac-1 activation is much lower than Rac-1 activation from CA-Rac expression. GLUT4myc myoblasts were transfected with WT- or GFP-CA-Rac, then stimulated with/without insulin for 10 min, followed by assaying Rac-1 activation using the pulldown assay. Pulled down GTP-Rac was detected with anti-GFP antibody.

4.3-2: Acute activation of Rac-1 is also capable of eliciting an insulin-like increase in basal surface GLUT4

While overexpression of CA-Rac and Tiam1 served as good models of activating Rac-1 alone, their effects more closely resembled the consequence of a “chronic” Rac-1 activity in the span of 24h post-transfection. In contrast, insulin induces GTP loading of Rac-1 within 1 min of addition and promotes maximal activation by 10 min (175,176). Therefore, to explore if the gain in basal surface GLUT4 observed with chronic Rac-1 activation would be recapitulated in an acute manner, we took advantage of a rapamycin-inducible heterodimerization system (264). The cell permeating rapamycin has high affinity towards the FK506-binding protein (FKBP), that within cells interacts tightly with the FRB domain of the protein kinase FRAP. Based on this principle, upon addition of rapamycin, a synthetic construct consisting of the protein of interest fused to
FKBP can be rapidly recruited to FRB. FRB can further be localized to the plasma membrane via a genetically engineered targeting sequence of Lyn kinase. Because active Rac-1 is normally localized to the plasma membrane due to prenylation of its C-terminal CAAX motif (265,266), the rapamycin-inducible strategy can be used to investigate acute Rac-1 activation by either a) replacing the C-terminal CAAX motif on CA-Rac with FKBP so that it remains cytosolic until recruited to the membrane by rapamycin, or b) generating an FKBP-chimeric protein containing the DH-PH domain of Tiam1 (domain required for the GEF activity towards Rac) (216). Using these two approaches to also eliminate any possible confounding effect arisen from the 24h GFP-CA-Rac expression, we investigated the effect of acute Rac-1 activation on GLUT4 traffic.

As proof of principle, we first examined whether co-expression of Lyn-FRB and YFP-tagged FKBP (YF) constructs would allow recruitment of YF to the plasma membrane upon the addition of rapamycin. In rounded-up myoblasts, which retain signalling capability but also provide improved spatial resolution compared to adhered cells (187), YF remained cytosolic in DMSO-treated control (Figure 4-5). In contrast, after 10 min of rapamycin treatment, the YF signal began to redistribute to the plasma membrane while cells without Lyn-FRB co-expression did not display this membrane recruitment (Figure 4-5). Similar rapamycin-triggered movement to the periphery was observed with either YF-CA-Rac lacking the CAAX motif (YF-CA-Rac) or a YF-linked DH-PH domain of Tiam1 (YF-Tiam1) co-expressed with Lyn-FRB (Figure 4-5).
**Figure 4-5: Rapamycin triggers the redistribution of YF-probes towards the Lyn-FRB at the plasma membrane.** YF, YF-CA-Rac, or YF-Tiam1 were co-transfected with/without Lyn-FRB in myoblasts. Cells were treated with/without rapamycin for 10 min followed by: A) rounded-up assay and detecting the redistribution of the YFP signal. Representative images of 3 independent experiments are shown. Bar = 10 µm.

To ascertain that Rac-1 activation only occurred after rapamycin-triggered recruitment of YF-CA-Rac and YF-Tiam1 to the plasma membrane, we probed for the characteristic Rac-mediated membrane ruffles (262). In DMSO-containing controls, neither YF-CA-Rac nor YF-Tiam1 expression generated membrane ruffling (Figure 4-6A). However, upon rapamycin addition (10 min), cells displayed extensive actin remodelling, evinced by the accumulation of phalloidin-decorated F-actin at sites of membrane ruffles (Figure 4-6A). On the contrary, rapamycin did not cause actin rearrangement in myoblasts expressing YF or YF-DN-Rac with Lyn-FRB (Figure 4-6B). Despite YF-CA-Rac being mostly concentrated in the nucleus due to the absence of prenylation and the presence of a nuclear localization sequence at the C-terminus of Rac-1, the addition of rapamycin was still able to mobilize it towards the plasma membrane to initiate actin remodelling (Figure 4-5, 4-6). These results demonstrate the ability of rapamycin to selectively trigger acute Rac-1 activation in myoblasts by

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recruiting YF-CA-Rac and YF-Tiam1 to the plasma membrane when co-expressed with Lyn-FRB.

Figure 4-6: Co-transfection of Lyn-FRB and YF-CA-Rac or YF-Tiam1 allows rapamycin-inducible actin remodelling. A-B) YF, YF-CA-Rac, YF-DN-Rac, or YF-Tiam1 were co-transfected with/without Lyn-FRB in myoblasts. Cells were treated with/without rapamycin for 10 min followed by permeabilization and staining with rhodamine phalloidin to measure changes in F-actin. Representative images of 3 independent experiments are shown. Bar = 10 µm.

Utilizing this rapamycin-inducible Rac-1 activation strategy, we examined the changes in surface GLUT4 following 10 min of acute Rac-1 activation. As shown in Figure 4-7, expression of YF-CA-Rac did not alter the amount of surface GLUT4 detected in the DMSO-containing control. However, upon 10 min of rapamycin treatment, which provided a surge in Rac-1 activity, the corresponding surface GLUT4 was significantly elevated compared to neighboring untransfected cells. This gain in surface GLUT4 was also reproduced by expressing YF-Tiam1 in myoblasts to turn on endogenous Rac-1 following rapamycin treatment, while YF-expressing controls failed to exert any change in GLUT4 at the plasma membrane (Figure 4-7B). Interestingly, the rapamycin-triggered elevation in surface GLUT4 achieved in cells with YF-CA-Rac or YF-Tiam1 was comparable to that observed in YFP controls after insulin stimulation.
(Figure 4-7A,B). These data suggested that, similar to the overexpression of GFP-CA-Rac (Figure 4-1), acute Rac-1 activation was sufficient to drive an insulin-like response on GLUT4 translocation.

A)
**Figure 4-7: Rapamycin triggers GLUT4 translocation in muscle cells when co-expressed with Lyn-FRB and YF-CA-Rac or YF-Tiam1.** A-B) YF, YF-CA-Rac, or YF-Tiam1 were co-transfected with/without Lyn-FRB in myoblasts. Cells were treated with/without rapamycin for 10 min followed by measurement of surface GLUT4myc. Changes were compared to those in YFP-expressing myoblasts stimulated with/without insulin and quantified relative to the YFP basal signal (mean ± SE, *p<0.05). Representative images of 3 independent experiments are shown. Bar = 10 µm.

4.3-3: Rac-1 superactivation signals through PI3K, Akt and AS160 to increase surface GLUT4

Because Akt activation is a prerequisite for insulin-dependent GLUT4 translocation, we were surprised that Rac-1 superactivation would cause GLUT4 translocation, as in response to insulin both signals appear to dissociate downstream of PI3K (147,177). Hence, we hypothesized that Rac-1 superactivation might potentially exert signalling cues to Akt and/or to the upstream canonical insulin signalling molecules IRS-1 and PI3K. To start, we analyzed whether IRS-1 is tyrosine phosphorylated upon Rac-1 superactivation. For these experiments, we used the chronic, sustained active Rac-1 approach. Myoblasts were co-transfected with HA-tagged IRS-1 and GFP-CA-Rac (or GFP as control), then IRS-1 was immunoprecipitated via its HA epitope and the corresponding level of P-tyr (pY) was examined with anti-pY antibody. When GFP was co-expressed with HA-IRS-1, the basal level of pY was negligible while insulin stimulation visibly enhanced pY in IRS-1 (Figure 4-8). IRS-1 immunoprecipitated from myoblasts expressing GFP-CA-Rac did not show any increase in pY compared to the basal amount in GFP-expressing controls (Figure 4-8), implying that IRS-1 is not engaged by Rac-1 activation.
Figure 4-8: Overexpression of GFP-CA-Rac does not cause tyrosine phosphorylation of IRS-1. HA-IRS-1 was co-transfected with GFP or GFP-CA-Rac before stimulating myoblasts with/without insulin for 10 min, followed by immunoprecipitation using anti-HA antibody. Immunoprecipitates were stained with anti-P-tyr antibody to detect phosphorylation of IRS-1. Representative images of 3 independent experiments are shown.

Next we evaluated whether GFP-CA-Rac would increase the levels of PI(3,4,5)P_3 at the plasma membrane, as an index of PI3K activation. Changes in PI(3,4,5)P_3 at the cell periphery were assessed by transfecting a PI(3,4,5)P_3-reporter constituted by the PH domain of Akt linked to RFP (PH-Akt-RFP) (267) and imaging rounded-up myoblasts. In unstimulated cells expressing GFP, the PI(3,4,5)P_3-reporter remained mostly cytosolic and only a faint peripheral signal was observed (Figure 4-9A). Upon insulin stimulation, the signal at the plasma membrane was clearly evident, corroborating that the hormone triggers PI(3,4,5)P_3 production (Figure 4-9A). When PH-Akt-RFP was co-expressed with GFP-WT-Rac, its distribution pattern was similar to that displayed in unstimulated cells expressing GFP (Figure 4-9A). Notably, however, expression of GFP-CA-Rac caused a clear redistribution of PH-Akt-RFP towards the plasma membrane, albeit lower than that caused by insulin (Figure 4-9A). This enrichment of PH-Akt-RFP at the membrane was not due to the increase in membrane folding caused by Rac-dependent actin reorganization because co-expression of RFP alone with GFP-CA-Rac failed to exhibit a similar reporter accumulation (Figure 4-9C). Assessment of the ratio of the PH-Akt-
RFP signal at the plasma membrane over the cytosolic signal (PM/cyto) in each condition revealed that Rac-1 superactivation via GFP-CA-Rac expression elevated PI₃K production to approximately 20% of the maximal insulin response while GFP-WT-Rac had no effect (Figure 4-9B).

![Image](image_url)

**Figure 4-9**: Overexpression of GFP-CA-Rac causes the redistribution of PH-Akt-RFP to the plasma membrane. A-B) PH-Akt-RFP was co-expressed along with GFP, GFP-WT-Rac, or GFP-CA-Rac. Rounded-up myoblasts were generated and stimulated for 10 min with/without insulin, following which the redistribution of the PH-Akt-RFP to the membrane was determined. PM/Cyto ratio of RFP in each condition was quantified and is expressed relative to the % of the parallel, maximal insulin response, in units normalized to GFP-expressing control cells. C) Myoblasts were co-transfected with GFP-CA-Rac and RFP. Myoblasts were rounded-up and used to visualize the distribution of RFP signal. Representative images of 3 independent experiments are shown. Bar = 10 µm. (mean ± SE).

The small gain in PI₃K in cells with increased Rac-1 activity served as a potential gateway to study Akt activation. Thus, using myoblasts co-expressing HA-Akt and either the GFP, GFP-WT-Rac or GFP-CA-Rac, we investigated the activation status
of Akt by probing for phosphorylation at the Thr308 and Ser473 sites necessary for functional Akt (149,150,268). As expected, insulin stimulation significantly increased the phosphorylation of Akt at Thr308 and Ser473 compared to unstimulated cells co-expressing GFP (control) (Figure 4-10A). When GFP-CA-Rac was co-expressed, in the absence of insulin, the phosphorylation of Thr308 and Ser473 was elevated to 26% and 62%, respectively, of the maximal insulin response measured in parallel (Figure 4-10A,B,C). In contrast, expression of GFP-WT-Rac also induced a 35% enhancement in Ser473 phosphorylation relative to the maximal action of insulin, but with little if any gain in Thr308 phosphorylation (Figure 4-10A,B,C).

**Figure 4-10: Overexpression of GFP-CA-Rac induces a modest gain in Akt phosphorylation.** A-C) Myoblasts expressing HA-Akt + GFP, GFP-WT-Rac, or GFP-CA-Rac were stimulated with/without insulin for 10 min before immunoprecipitation with HA antibody. Immunoprecipitates were analyzed for p-Akt using anti-p-Thr308 or p-Ser473 antibodies. Changes in P-Akt/total HA-Akt in each condition were quantified and are presented as % of the parallel, maximal insulin response, in units normalized to the GFP-expressing control. Representative images of 3-4 independent experiments are shown. (mean ± SE).

To ascertain whether the Rac-driven PI3K and Akt participate in the insulin-independent GLUT4 translocation caused by Rac-1 superactivation, we examined the
ability of wortmannin (WM) and Akt inhibitor 1/2 (Akti1/2) to interfere with this response. For these experiments, we used the acute Rac-1 superactivation assay (rapamycin-triggered) to avoid confounding effects of prolonged action of inhibitors. As shown in Figure 4-11, pretreatment of myoblasts with WM or Akti1/2 lowered the effectiveness of rapamycin-induced acute Rac-1 superactivation to elicit the increase in surface GLUT4 by 58% and 40%, respectively. On the contrary, pretreatment with compound C (CC), at concentration previously shown to prevent AMPK activity (269), did not alter the rapamycin-triggered response, which suggests that AMPK does not participate in the Rac-1 superactivation-mediated GLUT4 translocation (Figure 4-11). In parallel, we assessed the effect of LB to explore if actin dynamics is required for acute Rac-1 superactivation to increase surface GLUT4. Figure 4-11 illustrates that, indeed, LB significantly reduced the GLUT4 response, showing that, as in the case of insulin stimulation, actin dynamics is an obligatory component of Rac-1 superactivation-driven elevation in surface GLUT4 (165,166).

Figure 4-11: Inhibition of PI3K, Akt, and actin remodelling by inhibitors impair the Rac-1 superactivation-driven GLUT4 translocation response. GLUT4myc myoblasts transfected with Lyn-FRB + YF-CA-Rac were pretreated with DMSO, 100nM WM, 10µM Akti1/2, 200nM LB, or 10µM CC for 20 or 30 min (Akti1/2 and CC) followed by 10 min rapamycin treatment in the presence of inhibitors. The changes in surface GLUT4myc
were quantified and illustrated as % of the maximal rapamycin-induced response, relative to DMSO-treated control cells. (mean ± SE, *p<0.05 vs DMSO). Representative images of 3-4 independent experiments are shown.

It is well documented that submaximal Akt phosphorylation is sufficient to transmit downstream responses, and that there is amplification in the Akt pathway leading to GLUT4 translocation (147,208,209,270). We therefore explored whether the modest increase in Akt phosphorylation caused by GFP-CA-Rac sufficed to propagate the signal down to AS160, an essential component for the net gain of surface GLUT4 in response to insulin. When FLAG-AS160 was co-expressed with GFP (control), insulin promoted AS160 phosphorylation as detected by the increase in reactivity with PAS (anti-phospho Akt substrate antibody) (Figure 4-12A). This gain did not occur in unstimulated cells co-expressing GFP-WT-Rac and FLAG-AS160, whereas a substantial FLAG-AS160 phosphorylation, equivalent to 70% of the maximal insulin response, was observed in cells expressing GFP-CA-Rac (Figure 4-12A,B).

**Figure 4-12:** AS160 is phosphorylated upon GFP-CA-Rac overexpression and is required for the Rac-1-induced gain in surface GLUT4. A-B) FLAG-AS160 was co-expressed with GFP, GFP-WT-Rac, or GFP-CA-Rac before immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were stained with PAS antibody to detect changes in AS160 phosphorylation. The intensity of PAS/total FLAG-AS160 in each condition was quantified and is depicted as % of the parallel, maximal insulin response, in units normalized to the GFP-expressing control. (mean ± SE). Representative images of 3 independent experiments are shown.
Finally, we explored the participation of AS160 in the response of GLUT4 to Rac-1 superactivation. As previously observed (154,230), overexpression of the AS160-4A mutant, with mutations in four key residues normally phosphorylated by Akt in response to insulin, dampened the insulin-stimulated elevation in surface GLUT4 in control cells (co-expressing GFP) (Figure 4-13A,B). However, the AS160-4A mutant co-expressed with GFP-CA-Rac, prevented the characteristic gain in surface GLUT4 exerted by Rac-1 superactivation (Figure 4-13A,B). In fact, AS160-4A had a quantitatively similar effect on the response of GLUT4 to either insulin or Rac-1 superactivation (achieving only 37% and 30% increases in surface GLUT4, respectively) (Figure 4-13B). Collectively, these results substantiate the involvement of PI3K, Akt and AS160 in Rac-1 superactivation-driven GLUT4 translocation.
Figure 4-13: Overexpression of FLAG-AS160-4A blocks the GLUT4 translocation induced by CA-Rac. A-B) FLAG-AS160-4A was co-transfected with GFP or GFP-CA-Rac followed by stimulation with/without insulin to measure changes in surface GLUT4. Quantification of each condition is presented as % of the parallel, maximal insulin response, normalized to GFP-expressing insulin-stimulated cells. (mean ± SE, *p<0.05). Representative images of 3 independent experiments are shown. Bar = 10 µm.

4.3-4: Rac-1 superactivation relieves defective GLUT4 translocation imposed by JNK and ceramide
Insulin resistance of muscle and of muscle cells is typified by a lower GLUT4 translocation in response to the hormone, consequently reducing glucose uptake (271,272). During high-fat feeding, muscle insulin resistance involves the gain in lipids and their derivatives, with resulting activation stress kinases such as JNK that in turn interfere with IRS-1 tyrosine phosphorylation. In particular, JNK activation causes IRS-1 phosphorylation at Ser307, thereby functionally uncoupling IRS-1 from the insulin receptor and promoting IRS-1 degradation (217,273,274). Because the GLUT4 translocation elicited by Rac-1 superactivation illustrated above bypasses a requirement for IRS-1, we hypothesized that this feature might be utilized to restore defective insulin downstream of IRS-1. We therefore reconstituted the JNK-mediated insulin resistance in myoblasts by overexpressing a constitutively active JNK construct (FLAG-CA-JNK) recently shown to cause insulin resistance in vivo due to aborted signalling at the level of IRS-1 (217). As a proof of principle, co-expression of FLAG-CA-JNK and HA-IRS-1 significantly reduced the total level of HA-IRS-1 and enhanced the relative amount of IRS-1 phosphorylation of Ser307 (Figure 4-14A). Under these conditions, FLAG-CA-JNK significantly lowered the insulin-induced gain in surface GLUT4, attesting to the establishment of insulin resistance (Figure 4-14B,C). Notably, however, FLAG-CA-JNK did not prevent the gain in surface GLUT4 elicited by GFP-CA-Rac (Figure 4-14B,C). This observation suggests that the signalling components responsible for Rac-1 superactivation-driven GLUT4 translocation are not hindered by active JNK. Hence, Rac-1 superactivation is not susceptible to inhibition by insults causing insulin resistance at the level of IRS-1.
Figure 4-14: Rac-1 superactivation triggers GLUT4 translocation response that bypasses the signal defects exerted by CA-JNK. A) HA-IRS-1 was co-transfected with FLAG or FLAG-CA-JNK followed by immunoprecipitation with HA antibody to detect changes in Ser307 phosphorylation and degradation of IRS-1. B-C) GLUT4myc myoblasts co-expressed with FLAG-CA-JNK + GFP or GFP-CA-Rac were stimulated with/without insulin followed by surface GLUT4 staining. Surface GLUT4myc in each condition was quantified and is presented as % of the maximal, parallel insulin response, normalized to GFP-expressing, insulin-stimulated cells. (mean ± SE, *p<0.05). Representative images of 3 independent experiments are shown. Bar = 10 µm.
We and others have previously shown that insulin resistance can also arise as a result of intracellular ceramide accumulation (whether exogenously supplied or driven by excess saturated fatty acids), and such insulin resistance involves signalling steps downstream of IRS-1 (177,275,276). Delivery of cell permeating C2-ceramide has been used to recapitulate this model of lipotoxic insulin resistance. C2-ceramide reduces the insulin-induced activation of both Akt and Rac-1 (177,275,276). To investigate the susceptibility of the Rac-induced GLUT4 translocation to C2-ceramide, we pre-treated myoblasts with C2-ceramide before subjecting them to acute Rac-1 superactivation. Unlike the DMSO-containing YF control, whose insulin-mediated GLUT4 translocation significantly dropped to 21% with C2-ceramide treatment, Rac-1-induced GLUT4 gain at the membrane was far less affected, as the response was still 71% of the maximal attainable response (Figure 4-15). Presumably, Rac-1 superactivation allowed for sufficient Rac-1 and Akt activity to drive a partial GLUT4 translocation, even in the presence of C2-ceramide. We surmise that Rac-1 superactivation may be a principle to be applied in the future to counteract, at least in part, the defective GLUT4 traffic imposed by excess lipid supply.
Figure 4-15: Rac-1 superactivation partially restores the C2-ceramide-induced GLUT4 traffic defect. GLUT4myc myoblasts co-expressing Lyn-FRB + YF or YF-CA-Rac were pretreated with DMSO or 50µM C2-ceramide (C2) for 2h followed by treatment with/without insulin or rapamycin before staining for surface GLUT4. Results in each condition were quantified and are represented as % of the maximal, parallel insulin response relative to DMSO-treated, YF-expressing insulin-stimulated cells. (mean ± SE, *p<0.05). n=3.
4.4: Discussion

In response to insulin, Rac-1 acts in concert with the Akt ➔ AS160 signalling cascade to enact the net mobilization of GLUT4 vesicles to the plasma membrane of muscle cells. It is proposed that Rac-mediated actin remodelling results in a dynamic submembrane tether that enriches GLUT4 vesicles and signalling molecules for subsequent fusion (162,177,187,188). Only a fraction of the total cellular Rac-1 complement is activated in response to insulin, based on the comparison of Rac-GTP pulled down from cells expressing WT-Rac and CA-Rac (Figure 4-4), or from the comparison of the level of Rac-1 activation in insulin stimulated cells vs GTPγS-treated lysates (175). While the Akt➔AS160 and the Rac-1➔actin arms of insulin signalling operate independently of each other, supported by the inability of Rac-1 knockdown and Akti1/2 to inhibit respective Akt phosphorylation (177) and Rac-1 activation (Figure 4-16), it has been reported that overexpression of constitutively active Rac-1 can promote GLUT4 translocation (181). However, the underpinning molecular mechanism was not explored, and this question prompted the present study.

Figure 4-16: Akti1/2 does not affect insulin-stimulated Rac-1 activation. Myoblasts were pretreated with DMSO or 10µM Akti1/2 for 1h followed by insulin stimulation for 10 min, followed by assaying Rac-1 activation with the pulldown assay. Pulled down proteins were resolved on SDS-10% PAGE and immunoblotted with anti-Rac-1 antibody. Rac-1-GTP signal was quantified and normalized to total Rac-1 cell lysate. (mean ± SE; *p<0.05 vs basal). Representative images of 4 independent experiments are shown.
Surprisingly, we found that Rac-1 superactivation led to activation of PI3K, Akt and AS160 and these signals, along with the ongoing Rac-mediated actin remodelling, mirror the typical response elicited by insulin stimulation resulting in GLUT4 translocation to the cell membrane. AMPK does not participate in this insulin-independent response because a) application of compound C did not impair the Rac-1 superactivation-induced GLUT4 translocation (Figure 4-11), and b) insulin stimulation to GFP-CA-Rac expressing cells did not cause an additional gain in surface GLUT4, contrasting with the previously reported additive effect of AMPK and insulin (277,278). Notably, Rac-1 superactivation restored the GLUT4 response in muscle cells rendered insulin resistant via JNK activation or ceramide delivery.

4.4-1: Rac-1 superactivation leading to PI\(_{3,4,5}\)P\(_3\) production, Akt and AS160 phosphorylation

4.4-1-I: PI\(_{3,4,5}\)P\(_3\) accumulation

The finding that PI\(_{3,4,5}\)P\(_3\) production was induced by Rac-1 superactivation (Figure 4-9A,B) was surprising because growth factor-dependent Rac-1 activation is linked to PI\(_{3,4,5}\)P\(_3\)-mediated recruitment and activation of Rac GEFs such as Tiam1, P-Rex, and Vav (279-281). Nonetheless, overexpression of CA-Rac and acute Rac-1 superactivation bypassed this prerequisite and each led to accumulation of a PI\(_{3,4,5}\)P\(_3\)-sensitive probe at the plasma membrane of muscle cells (Figure 4-9A,B), fibroblasts, and neutrophils without a need of stimulus (282,283). On the other hand, expression of CA-Cdc42 did not provoke significant PI\(_{3,4,5}\)P\(_3\) production (282), and this small GTPase was also unable to increase surface GLUT4 (Figure 4-2). Hence, a potential feedback mechanism to elevate PI\(_{3,4,5}\)P\(_3\) appears to be Rac-specific, but the molecular basis for
this step remains elusive. A potential explanation is an interaction between Rac-1 and PI3K. A pulldown of cytosolic proteins bound to GST-Rac-1 loaded with GTP was found to contain PI3K activity, and it was subsequently revealed that the p85 subunit of PI3K can interact with Rac-1 in a GTP-dependent manner in vitro (284). Because active Rac-1 is naturally anchored at the plasma membrane through prenylation, such interaction between Rac-1 and PI3K would bring PI3K close to the vicinity of its substrate PI_{(4,5)P_2} at the plasma membrane for Rac-mediated PI_{(3,4,5)P_3} production. However, in vivo evidence of this interaction during growth factor stimulation is lacking.

Another contributing factor for the increased PI_{(3,4,5)P_3} generation by Rac-1 superactivation could be the accumulation of active PI3K units in the Rac-induced branched cortical actin (162). Biochemical evidence supports a PI3K-actin filament interaction, as PI3K was enriched in detergent insoluble fraction containing polymerized F-actin in chemotaxic Dictyostelium discoideum cells, and its localization to membrane was lost after treatment with Latrunculin A (285). More importantly, the catalytic subunit p110 of PI3K accumulated at the site of remodelled cortical actin following insulin stimulation in muscle cells (162). These observations support a role for cortical actin in positioning PI3K close to the membrane. Indeed, when Rac superactivation-mediated actin remodelling was perturbed by actin disrupting agents, the consequent PI_{(3,4,5)P_3} production at the plasma membrane was blocked (282,286). This may explain why LB treatment was most effective at blocking the GLUT4 translocation induced by Rac-1 superactivation (Figure 4-11), as disrupting the cortical actin lattice would prevent Akt activation secondarily to impaired PI_{(3,4,5)P_3} production, along with the elimination of Rac-mediated actin remodelling.
4.4-1-II: Akt phosphorylation

Akt must be phosphorylated at both its activation site (Thr308) and its regulatory site (Ser473) in order to become fully active \((149,150)\). Thr308 phosphorylation provides partial activation, while Ser473 phosphorylation on its own allows minimal activity but contributes to potentiation of Akt activity and substrate specificity \((150,287-289)\). According to these findings, the phosphorylation of Akt at both Thr308 and Ser 473 must have activated the kinase, as reflected by the phosphorylation of its downstream target AS160 \((Figures\ 4-10A,\ 4-12A)\). On the other hand, although overexpression of GFP-WT-Rac in myoblasts contributed to slight elevation in Ser473 phosphorylation \((Figure\ 4-10C)\), no Thr308 phosphorylation was achieved, and there was no ensuing AS160 phosphorylation \((Figures\ 4-10B,\ 4-12A,B)\).

Phosphorylation of Akt at Thr308 is controlled by PDK1 \((149)\). The rise in PI\(_{3,4,5}P_3\) at the plasma membrane serves as a cue to recruit and enhance PDK1 activity. Because Akt is also enriched at the plasma membrane via its PI\(_{3,4,5}P_3\)-binding PH domain \((290)\), Akt and PDK1 may come in close proximity, favouring Akt phosphorylation. As seen in Figures 4-9A and B, the amount of membrane-bound PI\(_{3,4,5}P_3\) in GFP-CA-Rac expressing cells is equivalent to about 20% of the maximal insulin-dependent response. This modest gain correlates well with the 26% increase in phosho-Thr308 Akt achieved by Rac-1 superactivation, relative to the maximal insulin response, suggesting linear transmission of the PI\(_{3,4,5}P_3\) signal amplitude to PDK1→Akt \((Figure\ 4-10B)\). Consistent with this calculation, Thr308 phosphorylation of Akt is considered the limiting determinant for Akt activation \((287,288,291,292)\).

On the other hand, mTORC2 is the kinase responsible for Ser473 phosphorylation of Akt in response to insulin \((293)\). mTORC2 exists in a complex
containing mTOR, mLTST8, and Rictor; however, the mechanism of mTORC2 activation is unknown (294). Recently, it was revealed that Rac interacts with mTOR in the mTORC2 complex in a GTP-independent manner (295). This interaction provides spatial regulation to mTORC2, as enrichment of active Rac at the plasma membrane would bring along mTORC2. Accordingly, Rac-1 activation by CA-Rac-GTP may position mTORC2 near the plasma membrane, where Akt is recruited by PI(3,4,5)P_3. The close proximity of mTORC2 and Akt might contribute to the increase in phospho-Ser473 Akt observed with Rac-1 superactivation without the need of insulin stimulation (Figure 4-10A,C).

In addition to PDK1 and mTORC2, the kinase PAK1 can phosphorylate Ser473 of Akt in vitro (296). Interestingly, PAK1 inhibition reduces IGF-1 stimulated Akt activation and the Akt hyperactivity of cancer cells (296-298). PAK1 can also act as a scaffold for both PDK1 and Akt (299). Because PAK1 is recruited through its CRIB domain to GTP-loaded Rac-1, an association of PAK1, PDK1, and Akt at the plasma membrane would provide an additional avenue, independent of PI(3,4,5)P_3 production, driving Akt phosphorylation following Rac-1 activation.

Because WM and Akti1/2 prevent the production of plasma membrane PI(3,4,5)P_3 and sterically bind to the PH-domain of Akt respectively (300), both agents impair Akt activation by hindering the recruitment of Akt to the plasma membrane. However, the above mentioned PAK1-Akt-PDK1 complex and direct Rac-mTORC2 interaction could alleviate the complete reliance on the traditional membrane PI(3,4,5)P_3 and PH-domain of Akt to trigger Akt activation. This could explain why only partial inhibitory effect was observed with WM and Akti1/2 on the Rac-1 superactivation-induced GLUT4
translocation (Figure 4-11). Alternatively, the extensive actin remodelling generated during Rac-1 superactivation could be the main driver of this insulin-like translocation response. Because remodelled actin can act as scaffold to position insulin signaling molecules such as Akt close to the plasma membrane (162), the inhibitory effect of WM and Akti1/2 on Akt recruitment would be reduced compared to the normal insulin stimulation. This scenario is consistent with the near complete reduction of rapamycin-triggered GLUT4 translocation with LB (Figure 4-11).

4.4-1-III: AS160 phosphorylation

In spite of the low level of Rac-1 superactivation-mediated Akt activation relative to the maximal insulin response (26%), downstream phosphorylation of AS160 amounted to approximately 70% of the maximal insulin response (Figure 4-12B). This signal amplification from Akt to AS160 has also been recently appreciated for the insulin signalling cascade. Insulin dose-dependence and Akt inhibitor studies in adipocytes and muscle cells suggest that very little Akt activity is required for near maximal AS160 phosphorylation (208,209). From those studies, 30% insulin-stimulated phospho-Thr308 Akt corresponds to approximately 80% of maximal phospho-AS160, approximating our measurements of phospho-Thr308 and phospho-AS160 in response to GFP-CA-Rac (Figure 4-12B). Moreover, we have shown that 19±10% Akt activity suffices for maximal GLUT4 translocation in insulin stimulated muscle cells (147,270) and similarly, a WM-mediated reduction in phosphor-Thr308 Akt to 20% of the maximal value allowed for greater than 50% of the maximal GLUT4 translocation (210). These observations reinforce the concept that Akt phosphorylation/activity does not correlate linearly with insulin-mediated GLUT4 translocation, but rather the phosphorylation status of AS160 closely matches GLUT4 translocation. These calculations are in good agreement with
the corresponding responses of Akt, AS160 and GLUT4 to Rac-1 superactivation observed in the present study.

4.4-2: Rac-1 superactivation overcomes insulin resistance

The level of Rac-1-GTP achieved in response to either chronic expression of GFP-CA-Rac or acute superactivation via rapamycin is likely to be much higher than the insulin-mediated activation of endogenous Rac-1 (Figure 4-4). siRNA-mediated silencing of endogenous Rac-1 does not affect insulin-mediated Akt activation, suggesting that Rac-1 activation does not influence Akt during normal insulin stimulation (177). This may be due to the low level of Rac-1 activation and the high level of Akt phosphorylation evoked by the hormone. Much higher levels of Rac-1 activity in the absence of hormonal stimulation, such as those imposed by the chronic and acute strategies described here, are required to visualize the Rac-mediated Akt activation. Nonetheless, Rac-1 superactivation suffices to elicit enough Akt activation to trigger an insulin-like GLUT4 translocation in muscle cells. This feature was explored in this study to counteract the effect of strategies that cause insulin resistance.

Obesity is a leading factor in the development of insulin resistance because of the lipotoxic and low-grade inflammatory environment created by excessive accumulation of saturated fatty acids (271,301). Both saturated fatty acids and inflammatory cytokines impact on skeletal muscle to activate stress kinases, including JNK, leading to IRS-1 serine phosphorylation and degradation (273,302). When this JNK-induced blockage of IRS-1 signalling was reproduced via overexpression of active JNK, insulin-dependent GLUT4 translocation was significantly diminished (Figure 4-14B,C). However, active JNK did not preclude the GLUT4 translocation evoked by Rac-1 superactivation (Figure 4-14B,C), ostensibly because the latter response bypasses
the requirement of IRS-1. Therefore, in vivo, when defects at the level of IRS-1 arise, Rac-1 activation may be a gateway to trigger downstream signalling of Akt and AS160 to drive a full GLUT4 translocation response.

Unlike JNK activation, intracellular ceramides produced from saturated fatty acid excess can directly inhibit insulin signalling downstream of IRS-1 (205,275,276,303). Ceramides can inactivate Akt without influencing IRS-1 or PI3K by both preventing recruitment of Akt to the plasma membrane and increasing PP2A activity that dephosphorylates Akt (304,305). In muscle cells, C2-ceramide not only reduces Akt but also insulin-dependent Rac-1 activation, while IRS-1 tyrosine phosphorylation remains intact (177). Interestingly, C2-ceramide only exerted a 29% reduction in superactivated Rac-1-driven GLUT4 translocation compared to a 78% decrease in GLUT4 insulin response (Figure 4-15). This suggests that Rac-1 superactivation provides enough surge in both Rac-1 and Akt activities to partially overcome the barrier imposed by ceramide, and hence averts the negative effects of the lipid metabolite.

Although reduced insulin-dependent glucose uptake is a key factor in the development of whole-body insulin resistance leading to type 2 diabetes, none of the currently treatments of the disease (metformin, sulfonylureas, and dipeptidyl peptidase inhibitors) acts directly on muscle, targetting instead hepatic glucose production and insulin secretion. Here we identify that Rac-1 activation alone suffices to trigger an insulin-like GLUT4 translocation without insulin stimulation (Figure 4-17). Unlike insulin, Rac-1 superactivation bypasses IRS-1 yet triggers signalling through PI3K, Akt and AS160. This response is resistant to the deleterious effects of JNK activation or ceramide accumulation, which cause insulin resistance. IRS-1 is a common target of agents and
conditions causing insulin resistance (271,272), hence improving insulin action downstream of IRS-1 should be considered strategically advantageous to improve metabolic outcomes, compared to approaches that require IRS-1 participation. Compounds that would turn on a Rac-specific GEF or inhibit Rac-specific GAP activity in time and tissue-selective manner would be particularly useful tools to design therapies for insulin resistance. In the future, strategies that could enhance Rac-1 activation to a more physiological level that is comparable to insulin stimulation will reveal additional insight about the potential of this concept of Rac-1-mediated insulin-like response.

Figure 4-17: Model of Rac-1 superactivation-induced GLUT4 translocation in muscle cells. *Left:* Normal insulin response observed in cells expressing endogenous levels of Rac-1. *Right:* Rac-1 superactivation promotes membrane accumulation of \( \text{PI}_{(3,4,5)} \text{P}_3 \), which results in moderate phosphorylation of Akt and significant phosphorylation at AS160. These signalling components together with actin remodelling satisfied the molecular requirements for GLUT4 translocation without input of insulin.
CHAPTER 5: Summary and future directions
5.1: Summary

Since the cloning of GLUT4 from insulin-responsive tissues, extensive efforts have been devoted to elucidate the complex molecular events leading to the insulin-induced GLUT4 mobilization from intracellular compartments to the plasma membrane. Much excitement was generated when AS160 was discovered as the Akt effector that controls the activation of Rabs (154). Because Rabs are the master regulators of vesicle traffic, this mechanism provides a direct signalling cue to the mobilization of GLUT4 vesicles. What is equally exciting is the appreciation of Rho GTPase-controlled actin reorganization in the overall GLUT4 translocation process. In muscles, the contribution of actin dynamics to insulin-mediated GLUT4 traffic was demonstrated with the use of actin disrupting agents such as LB, CD, and jasplakinolide in cell culture and isolated muscle fibers (161,165-167). Experiments using dominant negative mutants or siRNA-mediated knockdown demonstrate that this insulin-induced actin remodelling event is Rac-1-dependent in muscle cells (161,177). Recent muscle-specific Rac-1 knockout mouse models have recapitulated these in vitro findings and cemented the important role of Rac-1 in the outcome of insulin-stimulated GLUT4 translocation in muscles (178,179). Nonetheless, questions about the downstream effectors governing the Rac-1-induced actin dynamics and the effect of additional Rac-1 activity towards the GLUT4 traffic response remain unexplored.

In chapter 3, Arp2/3 and cofilin are identified to act downstream of Rac-1 to regulate the dynamics of insulin-stimulated actin remodelling. In agreement with its principal property of F-actin assembly, Arp2/3 initiates the actin branching responsible for the formation of the remodelled actin while cofilin balances out the burst in actin
assembly through its severing and depolymerizing activity. This dynamic regulation must be maintained in order to achieve proper actin remodelling for the traffic of GLUT4 vesicles to the plasma membrane because perturbation of either molecule significantly reduces the insulin-mediated gain in surface GLUT4 in muscle cells. This concept of actin dynamics governing membrane protein traffic to plasma membrane is not limited to GLUT4 as cofilin-regulated actin dynamics is also necessary in the gain of AMPA receptors on the plasma membrane of hippocampal neurons (306).

In both cases, stimulus-induced cofilin dephosphorylation is the prerequisite for the increase in severing and depolymerizing activity. The phosphorylation status of cofilin is the result of an intricate balance between LIMK and slingshot phosphatase. Although insulin activates LIMK through the Rac->PAK pathway, the overall response is the dephosphorylation of cofilin, which suggests the outweighing phosphatase activity from slingshot. This enhanced slingshot action is impinged upon the formation of remodelled actin as binding to F-actin significantly augments its phosphatase activity (239,307). To minimize slingshot's function in the basal state, protein kinase D has been shown to exert inhibitory phosphorylation on slingshot that prevents its interaction with F-actin and directly reduces its phosphatase activity (308,309). Interestingly, intrinsically active GSK can also inhibit slingshot activity through phosphorylation (310). Because GSK is turned off during insulin stimulation by activated Akt, this GSK regulatory mechanism could synergize with remodelled actin to increase the dephosphorylating capacity of slingshot on cofilin.

A defect in cofilin dephosphorylation upon insulin stimulation has recently been linked to impaired GLUT4 translocation and glucose uptake in skeletal muscles from
PAK1 knockout animals (311). Although the contribution of PAK1 towards cofilin dephosphorylation is unknown at the moment, it nonetheless provides the first suggestive evidence in vivo for a role of cofilin in the insulin-induced GLUT4 translocation response.

With the emergence of Rac-1 as a key component of GLUT4 traffic following insulin stimulation of muscles, experiments were designed to examine the influence of additional Rac-1 activity towards the translocation response. In chapter 4, we found an insulin-independent gain in surface GLUT4 upon overexpression of CA-Rac in myoblasts. This phenotype can also be achieved via acute Rac-1 activation. We further demonstrated the molecular principle behind this Rac-1-driven response by revealing the concomitant activation of Akt and AS160, which together with active Rac-1 provides the two independent signalling arms required for insulin-stimulated GLUT4 translocation in muscle cells. This unique signalling capacity induced by excess active Rac-1, which bypasses the requirement for IRS-1, was applied to evade the signalling defects imposed during stress kinase- and ceramide-induced insulin resistance. Moreover, this in vitro finding can be reproduced with isolated gastrocnemius muscle fibers overexpressing CA-Rac, suggesting similar molecular response is achievable in vivo (178).

Recently, Nozaki et al. proposed that RalA is an important downstream effector of Rac-1 in its insulin-independent action on GLUT4 translocation. Overexpression of CA-Rac in muscle cells causes the GTP loading of RalA and down-regulation of RalA dampens the CA-Rac-mediated gain in surface GLUT4 (312). Nonetheless, a mechanism for Rac-dependent RalA activation is not proposed. The activation of RalA
following insulin stimulation has been demonstrated in adipocytes and its GTP-bound state is achieved through Akt-dependent inhibitory phosphorylation on the Ral GAP complex (313). If a similar molecular mechanism holds true in muscle cells, the modest activation of Akt induced by active Rac-1 could contribute to the observed RalA GTP-loaded status. This would suggest that the CA-Rac-induced effect on RalA is mediated by Akt. Therefore, RalA is most likely downstream of Akt instead of Rac-1 in the overall insulin signalling cascade leading to the increase in surface GLUT4 in muscle cells.

Rac-1 has also been linked to contraction-induced glucose uptake in skeletal muscles (314). However, the effector which Rac-1 employs to achieve this response is unknown at the moment. Uncovering new players in this Rac-1-dependent glucose uptake during muscle contraction could shed insight into other contributing molecules that might also function in the Rac-1-induced GLUT4 translocation in muscle cells.
5.2: Future directions

5.2-1: Regulation of Rac-1 activity during insulin stimulation

5.2-1-I: Activation by GEFs

Although Rac-1 and its subsequent actin remodelling response were characterized following insulin stimulation in muscle cells, little exploration has been made to discover the regulatory mechanism governing its GTP-loaded status. Studies thus far indicate that the activation is PI3K-dependent because wortmannin treatment in muscle cells partially blocks the insulin-mediated GTP loading of Rac-1 (175,312). Given that GTPases are turned on by GEFs and many GEFs for Rho GTPases function downstream of PI3K (315), GEFs appear to be logical candidates responsible for the insulin-induced Rac-1 activation. One report proposes that FLJ00068 is the Rac-GEF during insulin stimulation but the lack of direct evidence illustrating impaired Rac activation with FLJ00068 knockdown invites more thorough examination before a definitive conclusion is reached.

Analysis of the existing literature for the criteria of PI3K-regulated activity and induction of Rac GTP loading suggests the following GEFs are the most likely candidates: Vav, Tiam, P-Rex, SWAP-70, SOS, α-PIX, β-PIX, and DOCK180. Preliminary semi-quantitative PCR analysis illustrates the expression of Vav-2, Vav-3, Tiam1, SWAP-70, SOS-1, SOS-2, α-PIX, β-PIX, DOCK180 and the proposed FLJ00068/PLEKHG in L6 myoblasts (Figure 5-1).
Figure 5.1: Expression profile of candidate Rac-GEFs in L6 myoblasts measured by semi-quantitative PCR. Primers and PCR cycles used are described in the method section.

This presents a scenario in which functional redundancy may occur because of the large number of GEFs with Rac GTP-loading activity in muscle cells. For example, although Vav-2 is tyrosine phosphorylation upon insulin stimulation to relieve itself from the autoinhibitory state (316), down-regulation of Vav-2 does not affect the net activation of Rac-1 by insulin in myoblasts (Figure 5-2A,B,C,D).
Figure 5-2: Vav-2 undergoes insulin-induced tyrosine phosphorylation but does not influence insulin-dependent Rac activation in myoblasts. A-B) Confluent myoblasts grown in 10cm diameter dishes were treated with/without insulin before subjecting to cell lysate preparation and immunoprecipitation with anti-Vav-2 antibody. The precipitate was resolved on a gel and blotted for tyrosine phosphorylation by P-tyrosine antibody. Changes in P-tyrosine were expressed relative to the basal state. C-D) Vav-2 was down-regulated by siRNA before subjecting to insulin-stimulated Rac activation assay. Quantification was made relative to active Rac in the basal state. Representative images of >3 experiments are shown. (mean ± SE, *p<0.05).

On the contrary, siRNA-mediated knockdown of Tiam1 reduces the insulin-stimulated Rac-1 activation (Figure 5-3A-B). This reduction is partial which implies other GEFs could also contribute to the net result of GTP-loaded Rac-1.

Figure 5-3: Tiam1 knockdown reduces insulin-mediated Rac activation. A-B) Tiam1 expression was down-regulated in myoblasts with siRNA before subjecting to insulin-induced Rac activation. Quantification was made relative to the basal state. Representative images of 6 independent experiments are shown. (mean ± SE, *p<0.05).

Sorting out the GEFs involved in this process will probably require down-regulation of multiple GEFs at the same time to achieve a complete impairment in Rac-1 activation following insulin stimulation. Nonetheless, the identification of Tiam1 serves as a starting point for future investigation. Additional experiments comparing the effect of FLJ00068 and Tiam1+FLJ00068 double knockdown on the level of GTP bound Rac-1 would reveal the contribution of each during insulin response.
One other GEF that would be interesting to test is Trio. Its interaction with F-actin crosslinking protein, filamin A, determines the localization of its GEF activity towards Rac (317). Because filamin A can be enriched at the plasma membrane by associating with remodelled actin or binding to integrins/membrane glycoproteins, it directly positions Trio at the plasma membrane where Rac needs to be activated. Such molecular mechanism could be another method contributing to the burst of Rac-1 GTP following insulin stimulation.

5.2-1-II: Inactivation by GAP

The other side of the equation for Rac activation is the control of GAPs. Inhibition of Rac-GAP activity could also contribute to the net increase in Rac-1 GTP during insulin stimulation in muscle cells. Although the regulation of GAPs is less developed compared to GEFs, one intriguing notion has been the RhoA-dependent crosstalk to Rac through a Rac-GAP. Overexpression of CA-RhoA blocks nerve growth factor-induced Rac activation and Rac-dependent neurite outgrowth in PC12 cells and this inhibition is dependent on its downstream effector ROCK (318). It was subsequently revealed that ROCK phosphorylates FilGAP, which has GAP activity towards Rac, to enhance the hydrolysis of GTP-bound Rac (319). Interestingly, in parallel with nerve growth factor-stimulated Rac activation, the amount of GTP-associated RhoA was concomitantly decreased. It is therefore proposed that this chain of RhoA->ROCK->FilGAP signalling event towards Rac-GTP hydrolysis is hindered by the lower level of active RhoA so that a greater Rac activation is achieved following external stimulation. A similar mechanism could play a role in the insulin-mediated Rac-1 activation in muscle cells where the amount of GTP-loaded RhoA decreases upon insulin stimulation.
to reduce the GAP activity of FilGAP towards Rac-1. A time course measurement of RhoA activation during insulin treatment and examining the effect of CA-RhoA overexpression on Rac-dependent actin remodelling will provide the initial data to test this hypothesis.

5.2-2: The role of PAK in GLUT4 translocation

In addition to Arp2/3- and cofilin-driven activities, there are still several effector molecules that are controlled by Rac activation (320). With Rac-1 now being cemented as a critical component of insulin-induced GLUT4 translocation in muscles, examining different Rac-dependent signalling response in relation to the overall GLUT4 traffic could reveal a new Rac-induced mechanism. One of the common effectors regulated by Rac is PAK. PAK is relieved from its autoinhibitory state with the binding of active Rac and Cdc42 (321). In a PAK1 knockout mouse model, Wang et al. observed impaired insulin-mediated GLUT4 translocation in skeletal muscles which led to peripheral insulin resistance in the animal (311). However, the molecular mechanism by which PAK1 affects GLUT4 translocation remains elusive.

Previous studies on PAK have illustrated its ability to influence actin dynamics through the PAK->LIMK->cofilin pathway (80). At the same time, it can modulate the level of Rac activation by promoting the phosphorylation of GDI and GDI’s subsequent dissociation from Rac GTPase (322). Because proper activity of Rac-1 and actin dynamics are prerequisites for insulin-induced gain in surface GLUT4 in muscle cells, it can therefore be hypothesized that PAK regulates the insulin response via these two molecular mechanisms. Preliminary results utilizing IPA-3, a PAK inhibitor, in muscle
cells shows impaired insulin-induced Rac-1 activation and actin remodelling, which suggests PAK’s participation in Rac-regulated actin dynamics (Figure 5-4A,B).

![Figure 5-4](image)

**Figure 5-4: IPA-3 inhibits insulin-mediated Rac activation and actin remodelling.** Myoblasts were pretreated with DMSO or 30µM IPA-3 followed by 10 min of insulin stimulation before measurement of A) Rac activation, and B) actin remodelling. Representative blots and images of 2 independent experiments.

To evaluate the effect of PAK towards Rac activation via GDI, we can show that insulin stimulates the phosphorylation of GDI via detection with an antibody against PAK-specific phosphorylation site on GDI (Figure 5-5). Concomitant with the phosphorylation of GDI is a reduction in GDI-associated Rac-1, implying the release of sequestered Rac-1 and priming for GTP loading (Figure 5-5).
Figure 5-5: Insulin stimulates the phosphorylation of GDI and the release of Rac from GDI. Myoblasts were stimulated with/without insulin for 10 min before subjecting to immunoprecipitation with GDI antibody. The precipitates were subsequently analyzed with gel electrophoresis to reveal the changes in P-GDI and the level of GDI-Rac association. Representative blots of >6 independent experiments were shown.

These preliminary findings pave the groundwork to investigate PAK in the insulin-regulated response in muscle cells. It would be intriguing to apply IPA-3 to observe if it reduces the effect of insulin-stimulated phosphorylation of GDI caused by PAK. These findings will need to be validated with siRNA-mediated knockdown of PAK isoforms. In addition, PAK is a serine/threonine kinase whose phosphorylation capability on other common insulin signalling molecules with serine/threonine phospho-modulation has never been examined in detail. For example, AS160 has multiple serine and threonine phosphorylation sites that are for now attributed to Akt (154). However, it is equally possible that PAK may have an unestablished connection through direct AS160 phosphorylation. This hypothesis could be another explanation for substantial phosphorylation AS160 seen with CA-Rac overexpression. Uncovering the precise regulation induced by PAK downstream of Rac-1 activation would provide additional signalling cues that contribute to GLUT4 traffic.

5.2-3: Function of remodelled actin in GLUT4 traffic in muscle cells

The proposed mechanism of remodelled actin in GLUT4 traffic is largely derived from actin disrupting agents that harshly perturb whole cell actin dynamics. Furthermore, advanced TIRF microscopy studying the effect of remodelled actin has only been examined in adipocytes but not muscle cells (192-194). With the identification of Arp2/3 and cofilin in controlling the respective polymerization and depolymerization step during insulin-mediated actin remodelling without disrupting F-actin at the level of stress fibers,
we have the opportunity to examine the finer changes in GLUT4 vesicle traffic within the TIRF zone in muscle cells with downregulated Arp2/3 or coflin.

Because siRNA to Arp2/3 prevents the formation of insulin-stimulated remodelled actin and coflin knockdown produces cortical F-actin aggregates, a comparison of their impact on GLUT4 vesicle mobility, enrichment, and insertion beneath the plasma membrane via TIRF microscopy would differentiate the traffic defects in the absence of remodelled actin versus that in the presence of aggregated actin meshwork. The recently engineered pHluorin-GLUT4-TdTomato construct (193), which allows precise distinction of vesicle tethering and fusion events in the TIRF zone, would help clarifying the mode of GLUT4 traffic in muscle cells. Findings from such experiments would expand our knowledge on the function of the remodelled actin in GLUT4 traffic and address any similarities and differences in the contribution of remodelled actin towards GLUT4 mobilization between adipocytes and muscles.

5.2-4: Bridging microtubule and F-actin in insulin-stimulated GLUT4 vesicle traffic

The cytoskeleton input towards insulin-mediated GLUT4 traffic to the plasma membrane is not limited to cortical remodelled actin, and indeed microtubules have also been shown to participate in this mobilization event. GLUT4 containing vesicles migrate on fluorescent tubulin-decorated microtubules and this long range movement is inhibited upon the addition of microtubule disrupting agents such as nocodazole and colchicine (198,199,323). Furthermore, overexpression of a functionally dead mutant of kinesin, a microtubule motor protein, prevents the vesicle movement leading to reduced gain in surface GLUT4 on the plasma membrane of adipocytes (199). These observations have
led to the proposition that microtubule-dependent tracks are involved in long range guidance of GLUT4 vesicles towards the plasma membrane.

On the other hand, previous studies have also implicated myosin5, an actin motor protein, in mobilizing GLUT4 vesicles for the insulin-induced translocation event (159,197). Because the disruption of actin dynamics do not inhibit the arrival of GLUT4 vesicles near the plasma membrane (193,194), the actin-based motor process is most likely occurring at the region of cortical remodelled actin beneath the plasma membrane, which contrasts the long range vesicle movement controlled by microtubules. In order to coordinate the movement of GLUT4 vesicles on two cytoskeleton networks, it can be hypothesized that GLUT4 vesicles first travel on the microtubule network followed by the switch to the F-actin tracks when approaching the membrane. This crosstalk between microtubule and F-actin during vesicle traffic can be supported by the observation that kinesin and myosin5 interact with one another to potentially enable tract switching (324). This change in tracts can be visualized in vitro when beads are artificially attached with both actin and microtubule motors (325).

In the context of GLUT4 traffic, the switching model is reinforced by the lack of colocalization between the remodelled actin and microtubules at the cell cortex following insulin stimulation in myotubes (326). Instead, microtubules are clustered around remodelled actin which favours a need to change tracks for vesicles to reach to the plasma membrane. In order for the switching to occur efficiently, it can be imagined that the two cytoskeleton networks must be integrated closely together to facilitate the motor proteins to change tracks. This concept has led to the identification of linker proteins that bridge between microtubules and F-actin. Besides being an F-actin crosslinking
protein, IQGAP simultaneously associates with microtubule via its interaction with microtubule tip protein CLIP-70 (327). Combining IQGAP’s microtubule connection with its ability to bind to active Rac through its GTPase binding domain (327), IQGAP can potentially direct microtubule orientation towards the site of active actin remodelling to facilitate track-mediated vesicle movement towards the plasma membrane. More recently, another protein CLIPS2 was also shown to bridge IQGAP to microtubule and this interaction is prevented by GSK-3-dependent phosphorylation of CLIPS2 (328). Given that insulin-stimulated Akt activity can inactivate GSK-3 (329), this may promote the complex formation between CLIPS2 and IQGAP in order to link microtubule and F-actin at the cell cortex.

Although the participation of IQGAP-CLIP-70 or IQGAP-CLIPS2 in the GLUT4 traffic response has not yet been fully examined, a few existing evidences do support their role in insulin response. First, single nucleotide polymorphism of IQGAP is associated with the development of type 2 diabetes (330). Down-regulation of IQGAP in muscle cells would address whether IQGAP has a functional input in insulin-stimulated GLUT4 traffic. Secondly, CLIPS2 is serine phosphorylated in response to insulin and siRNA-mediated knockdown of CLIPS2 reduces insulin-induced GLUT4 translocation in myotubes (331). Nonetheless, the visualization of the cellular defect in GLUT4 traffic in relation to microtubule and F-actin that occurs following CLIPS2 down-regulation has yet to be examined. It would be intriguing to expand this study by comparing the microtubule-actin connection beneath the plasma membrane with IQGAP, CLIP-70, or CLIPS2 knockdown by TIRF microscopy. Capturing GLUT4 vesicles switching between microtubules and F-actin by TIRF would also provide first evidence supporting this track
switching hypothesis. Furthermore, the validation of serine phosphorylation on CLIPS2 by Akt following insulin stimulation would enable the test on its contribution to the complex formation with IQGAP and microtubule-actin linking capability.

Collectively, these proposed studies will lead to a better understanding of 1) how Rac-1 is activated in response to insulin in muscle cells, 2) how Rac-dependent PAK functions in insulin-stimulated GLUT4 translocation, 3) how modulation of actin dynamics influences the hypothesized functions of remodelled actin in GLUT4 traffic, and 4) how microtubules and actin filaments integrate to achieve the net mobilization of GLUT4 vesicles. This effort will introduce novel regulators and molecules to the existing insulin signalling cascade in muscle cells. The growth in understanding of molecular signals contributing to insulin-induced GLUT4 translocation will lay the basic groundwork necessary to dissect the varying molecular defects in the growing number of patients, families, and populations with insulin resistance. The ability to pinpoint with precision the molecules responsible for impaired GLUT4 translocation and consequent defective glucose uptake in muscles will assist in the targeted therapeutic design to combat insulin resistance. The significance of such findings cannot be underestimated, as there is currently few reliable treatment option targeted to reverse insulin resistance conditions. Type 2 diabetes is managed at present with drugs that promote insulin secretion (sulfonylureas, GLP-1 analogues or dipeptidyl peptidase IV inhibitors) or that reduce hepatic glucose output (metformin). There is a sore need to develop strategies that will relieve peripheral (i.e. muscle) insulin resistance, which is the first event leading to whole body insulin resistance and progression to type 2 diabetes.
CHAPTER 6: References


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