COORDINATION OF CELL SIGNALING AND TRANSPORT MACHINERY DURING INSULIN-STIMULATED GLUCOSE TRANSPORT

By

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TO

THOSE WHO MADE ME PROUD AS ONE OF THEM;

AND I SHALL MAKE THEM PROUD AS ONE OF US.

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Preface

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Abstract

Insulin-stimulated glucose transport is the rate-limiting step in glucose disposal and utilization. Insulin increases glucose uptake in fat and muscle through the translocation of the insulin-responsive glucose transporter Glut4 to the plasma membrane. Although our understanding of the pathways governing this process remain incomplete, small GTPases have been implicated as "molecular switches" that operate at the crossroads of insulin signaling and Glut4 translocation. This thesis elucidates the role of RalA, a small GTPase that regulates an octameric vesicle-tethering complex known as the exocyst during Glut4 trafficking.

Initial studies on the cell cycle revealed that both RalA and the exocyst are involved in trafficking through the recycling endosome. Loss of RalA or the exocyst led to a specific blockade in cell abscission, the very last stage of cytokinesis, implying the involvement of these proteins in a subset of transport events under tight regulation.

Glut4 traffics through the recycling endosome in adipocytes. We found that RalA resides on the Glut4 vesicles and interacts with the exocyst in insulin-responsive cells. Insulin activates RalA in a PI-3 kinase-dependent manner. Disruption of RalA function led to inhibition of insulin-stimulated glucose transport, as did loss of the exocyst. Furthermore, RalA also binds to Myo1c, a molecular motor previously implicated in Glut4 trafficking. This interaction is modulated by Calmodulin, which functions as the light chain for

Myo1c during insulin-stimulated glucose transport. The data suggested a dual role for RalA in insulin action, as a cargo receptor for Myo1c and a signal to unify the exocyst.

The architecture of the exocyst complex was further dissected with RalA mutants uncoupled from one branch of its effectors, including the two exocyst subunits Sec5 and Exo84. We found that both subunits are required for exocyst function in glucose transport; however, they belong to different branches of the exocyst complex that also contain overlapping subunits. Furthermore, three exocyst subunits Sec8, Sec6, and Sec5 form a sub-complex that targets RalA-localized vesicles.

Taken together, these data suggest that RalA integrates upstream signaling from the insulin receptor to mobilize downstream transport machineries, leading to the specificity required for the actions of insulin.

Chapter 1

Introduction

Diabetes Mellitus is a metabolic disorder characterized by elevated blood glucose and dyslipidemia. Chronic diabetes can be categorized into two major forms, type 1 and type 2. While both disorders present with hyperglycemia and a similar sequelae of complications, including increased risk of cardiovascular disease and stroke, renal disease, blindness, and neuropathy [1], their etiologies are unrelated. Type 1 diabetes, which only accounts for ~5-10 % of diabetes in the U.S., results from autoimmune destruction of pancreatic β-cells. In contrast, type 2 diabetes occurs due to a combination of reduced insulin sensitivity and insulin secretion. Type 2 diabetes is a leading cause of mortality and morbidity worldwide. There were an estimated 143 millions people affected by the disease by the year 2003 [1], and this number is projected to double to 300 million by the year 2025 [2]. Type 2 diabetes occurs over a continuum of worsening insulin resistance. Eventually the beta cells of the endocrine pancreas become unable to compensate for the increased demand for insulin, and a period of decompensation occurs, leading to impaired glucose tolerance, and eventually frank diabetes [3]. In this regard, insulin resistance normally precedes β-cell failure [4], making the former the first lesion in the pathogenesis of type 2 diabetes. Therefore, a better understanding of the molecular mechanisms of insulin action may help in the development of new therapeutic strategies for this disease.

Insulin is the most potent anabolic hormone known, promoting the storage and synthesis of lipids, protein, and carbohydrates and inhibiting the breakdown and release of these molecules into the circulation, thus produces a net gain in energy intake and storage [5]. In muscle and fat tissue, the first and rate-limiting step in this energy surplus involves facilitated glucose uptake; a process mediated by the glucose transporter Glut4 [6]. Insulin accelerates the endocytic recycling of Glut4 to the plasma membrane, thus stimulating gluocose clearance from the circulation.

THE INSULIN-RESPONSIVE GLUCOSE TRANSPORTER GLUT4

Glut4 (Figure 1.1), encoded by the gene SLC2A4, belongs to a family of facilitative sugar transporters that contain 12 transmembrane domains [6, 7]. These proteins catalyze hexose transport across the bi-layer cell membrane in an ATP-independent, facilitative diffusion manner [8]. Glut4 is the only family member that displays responsiveness to insulin stimulation by changing its cellular localization [7, 9]. Moreover, the tissue distribution, transcriptional control, as well as protein stability of Glut4 highly correlates with insulin signaling and its regulation of energy metabolism [7, 9]. Therefore, Glut4 represents a key player in maintaining whole body glucose homeostasis.

The key role played by Glut4 is evident in various mouse models in which Glut4 has been specifically ablated [10]. Whole body Glut4 knockout (Glut4 -/-) mice display growth retardation, with markedly reduced adipose mass, cardiac hypertrophy, and shortened life span [11]. Surprising, these mice do not develop diabetes, possibly due to

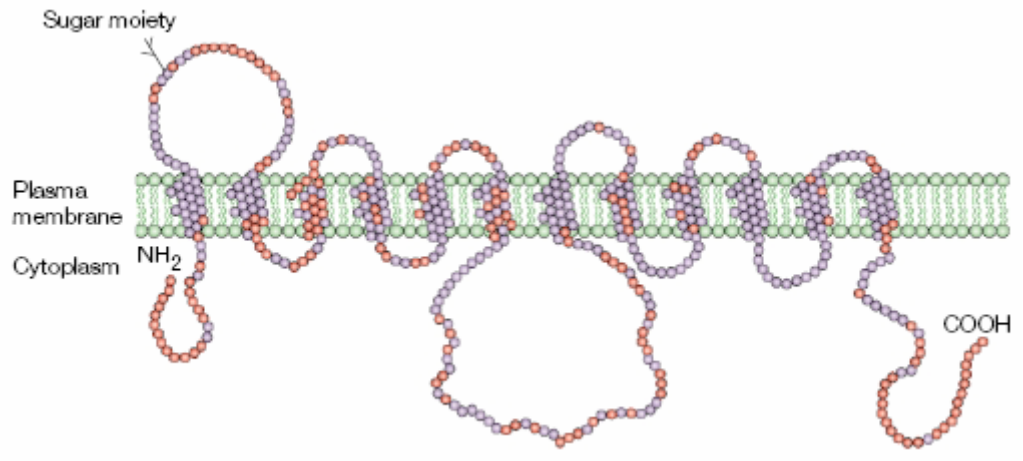

Figure1.1 Schematic view of the Glut4 transporter The GLUT family of proteins is comprised of 13 members, all of which are 12-span trans-membrane proteins with both amino- and carboxyl-termini located in the cytosol. Based on their sequence homology, these transporters have been catogorized into three subclasses: Class I (GLUTs 1–4) are glucose transporters; Class II (GLUTs 5, 7, 9 and 11) are fructose transporters; and Class III (GLUTs 6, 8, 10, 12 and HMIT1) are structurally atypical members of the GLUT family, whose properties and functions are poorly studied to date. The diagram shows a homology plot between GLUT1 and GLUT4. Residues that are unique to GLUT4 are shown in red. (Figure adapted from Byrant et al, Ref. 7)

compensatory mechanisms that are required to promote survival of these animals [11, 12]. Heterozygous Glut4-null (Glut4 +/-) mice develop insulin resistance with an increased tendency to become diabetic [13-15]. The role of Glut4 in glucose homeostasis is further supported by its conditional depletion from muscle or adipose tissue. Deletion of Glut4 in muscle, the organ primarily responsible for the largest amount of insulin-stimulated glucose disposal, causes insulin resistance and more frequent diabetes [16]. Intriguingly, loss of Glut4 in adipose tissue leads to a similar metabolic disorder [17], despite the fact that adipose tissue accounts for only a small portion of insulin-stimulated glucose clearance [18]. This indicates crosstalk between different organs in metabolic regulation [19-22], and further that Glut4 plays a role as a gatekeeper for energy sensing in adipocytes. Furthermore, adipose-specific transgenic over-expression of Glut4 in Glut4 +/- mice largely reverses the insulin resistant phenotype and prevents the development of diabetes [23]. Consistent with this, over-expression of Glut4 in skeletal muscle or adipose tissue on a wild type background results in improved insulin sensitivity and glucose tolerance [24, 25]. Taken together, these genetic models implicate a pivotal role of Glut4 in whole body glucose metabolism.

CELLUAR MACHINERIES THAT PARTICIPATE IN GLUT4 TRAFFICKING

Given the importance of Glut4 in glucose homeostasis, much attention has focused on the molecular mechanisms by which insulin regulates the function of this protein. Insulin increases glucose uptake mainly by producing the translocation of the Glut4 proteins to the plasma membrane, rather than by increasing the intrinsic activity of the transporter

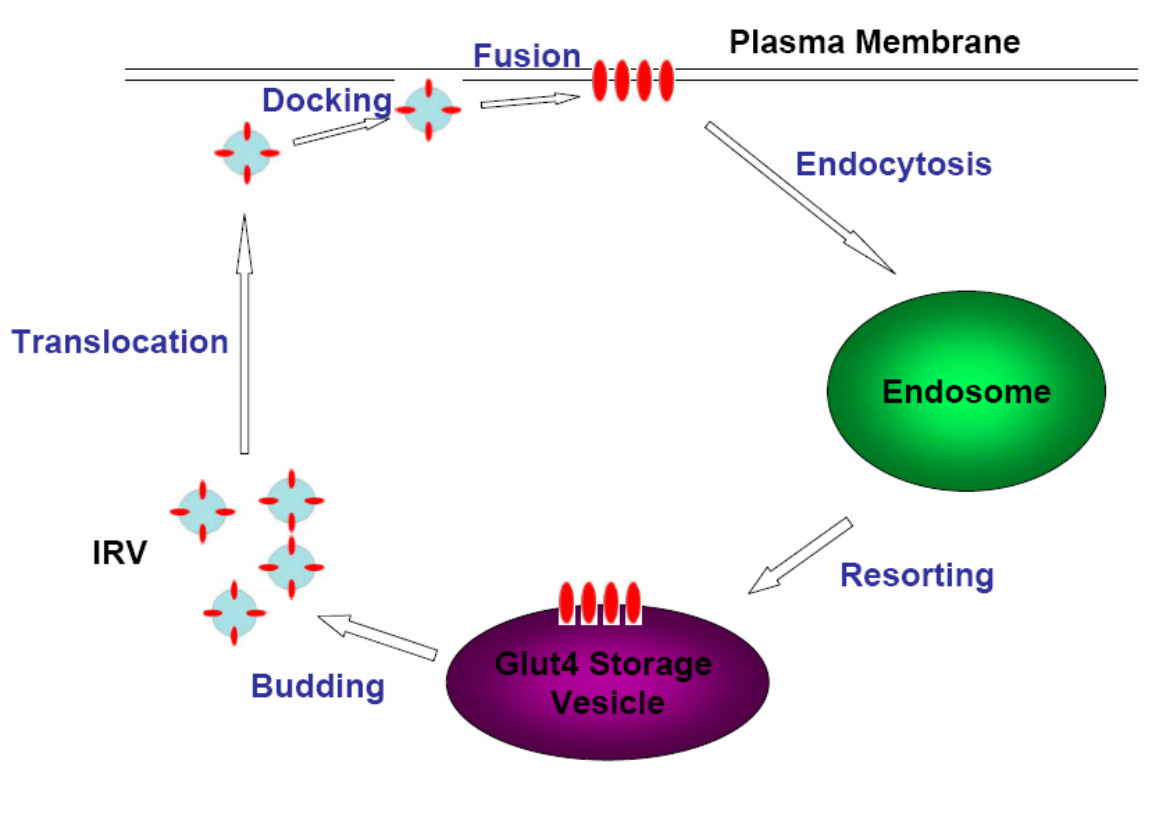

Figure 1.2 Itinerary of Glut4 trafficking In the basal states, Glut4 resides on the intracellular Glut4 Storage Vesicles (GSVs), which undergo dynamic exchanges with the recycling endosome (RE) and/or the trans-Golgi-network (TGN). The insulin-responsive Glut4 vesicles (IRVs) are thought to bud directly from GSVs or the recycling endosome (RE), a step that may involve the TGN as well. Upon activation of the insulin receptor by insulin, these insulin-responsive Glut4 vesicles undergo a rapid and robust re-location towards the plasma membrane. These Glut4 vesicles then dock and fuse with the plasma membrane, resulting in extracellular exposure of Glut4 and thus glucose uptake into cells. Glut4 on the cell surface then undergoes endocytosis via a clathrin-mediated process, and undergoes resorting to re-generate the insulin-responsive Glut4 vesicles.

[5, 26]. Furthermore, Glut4 trafficking represents one of the best studied examples of endocytic recycling of membrane proteins (Figure 1.2) [7, 27, 28]. In the basal state, Glut4 is largely retained in intracellular vesicles, which may undergo a futile cycle with the endocytic compartments and perhaps the trans-golgi-network (TGN) [7]. The insulin-responsive Glut4 vesicles are thought to bud directly from the recycling endosome (RE) [27], a step that may involve the TGN as well [7]. Upon activation of the insulin receptor by insulin, these insulin-responsive Glut4 vesicles undergo a rapid and robust re-location towards the plasma membrane, a step involving molecular motors of the kinesin and myosin family [6]. The Glut4 vesicles then dock and fuse with the plasma membrane, resulting in extracellular exposure of Glut4 and efficient glucose uptake into cells [5, 29, 30]. Glut4 on the cell surface then undergoes endocytosis via a clathrin-mediated process, and resorted to re-generate the insulin-responsive Glut4 vesicles [26, 30]. In general, insulin accelerates the exocytosis rate of Glut4 by 10-20 fold, while slightly decreasing the endocytosis rate of Glut4 by 1-2 fold, in the process leading to a large increase of plasma membrane-localized Glut4 [6].

Composition and Biogenesis of Intracellular Glut4 Storage Compartments

The first lines of evidence indicating the translocaton of Glut4 came from the reports that insulin causes re-distribution of "glucose transport activity" from intracellular compartments to the plasma membrane [31, 32]. This idea was certainly supported when five independent groups identified and cloned the insulin-responsive glucose transporter Glut4 in 1989 [33-37]. To date, compelling evidence has suggested that Glut4 is localized on intracellular membrane compartments referred to as Glut4 storage vesicles (GSV), and

further sorted into specialized insulin-responsive vesicles (IRV). The latter population of vesicles then translocate to the plasma membrane upon insulin stimulation [7]. However, the exact identities and properties of the GSVs or IRVs remain elusive, largely due to the dynamic nature of these membrane compartments [27, 38].

Early studies compared membrane compartments containing Glut4 with vesicles characterized in the secretory pathways, particularly since adipocytes also release several classes of secretory proteins in response to insulin [39, 40]. However, none of the proteins studied, including adipsin, leptin, lipoprotein lipase, and adiponectin, displayed functional overlap with Glut4 vesicles [41-45], reflecting the differences in trafficking routes between trans-membrane proteins such as receptors or transporters and soluble secretory proteins [28]. Indeed, many trans-membrane proteins undergo endocytic recycling that enables efficient recycling of the existing proteins after exocytosis [27, 28], while secretory proteins normally utilize the bio-synthetic route to load the vesicular carriers for release to the extracellular environment [28]. Consistent with this notion, Glut4 is still efficiently internalized in the presence of insulin, and only ~30-50% of total protein is present on the plasma membrane in this state [7, 27].

The insulin responsive Glut4 vesicles are thought to be generated from either the recycling endosome or the trans-golgi network [46-50], and undergo a futile cycle with these compartments in the basal state [7, 27]. A portion of Glut4 vesicles contain endosome markers such as the Transferrin Receptor (TfR) and VAMP3 [47, 48], while others contain TGN markers such as Syntaxin6 or Syntaxin16 [51-53]. However, further

dissection of the trafficking itinerary of Glut4 at this level has been unsuccessful, possibly due to the overlapping and dynamic nature of these membrane compartments [7]. Nevertheless, several elegant studies using compartment ablation have found that a portion of Glut4 is packaged into specialized vesicle populations that are segregated from the general endocytic or TGN vesicles [54-56].

Although the exact composition of insulin-responsive Glut4 vesicle remains to be characterized, a number of proteins have been reported to be integral components of these vesicular compartments [57, 58]. Among them, the insulin-regulated amino peptidase (IRAP) has been characterized as a major IRV protein [59-62]. IRAP is a 165 Kd type II trans-membrane protein with a 109-amino acid long cytosolic tail at its N-terminus [63]. IRAP has been shown to cleave vasopressin, oxytocin, lys-bradykinin, met-enkaphalin, dynorphin, and angiotensin III and IV, and possibly work as an angiotensin IV receptor [63, 64]. Interestingly, IRAP-null mice show decreased Glut4 vesicles and Glut4 protein levels in various tissues including adipose tissue, muscle, and heart [65]. This suggests a possible role of IRAP in biogenesis of Glut4 vesicles or maintaining the integrity of these vesicles. In addition, IRAP has been shown to directly interact with the Akt kinase substrate AS160 [66, 67], a protein involved in Glut4 vesicle trafficking [68]. Nevertheless, IRAP-null mice appear to have normal glucose metabolism, suggesting potential compensatory mechanisms for the loss of this protein [65].

Sortilin is another major protein component of Glut4 vesicles [48, 69, 70], and has recently been shown to be both essential and sufficient to generate insulin-responsive

Glut4 vesicles [71]. Sortilin is a type I membrane receptor containing an N-terminal furin cleavage site, a trans-membrane domain [72], and an acidic C-terminal luminal domain that is homologous to that of the yeast vacuolar protein sorting receptor, VPS10 [73-75]. Intriguingly, this intra-luminal VPS10 domain of sortilin appears to play a predominant role in targeting to the Glut4 vesicles [71]. Furthermore, the role of sortilin in generating Glut4 vesicles may involve its interaction with the Golgi-localized γ-ear-containing Arf-binding proteins (GGA) of coat adaptor proteins [30, 76, 77], although GGA proteins have only been suggested to play a role in sorting of newly-synthesized Glut4 [30, 78, 79]. On the other hand, sorting of recycled Glut4 may involve AP-1 adaptor proteins [80, 81] or ACAP1 and coat proteins such as clathrin [82].

Compared to vesicle carriers such synaptic [83] or COPI/COPII vesicles [84, 85], GSVs or IRVs remain poorly characterized. One would predict that these vesicles contain phosphatidylinositol phosphates and enzymes that modify these lipids, small GTPases of Rab or Arf family, SNARE proteins, and target proteins of insulin signaling [83-85]. However, the function of most of these molecules in regulating Glut4 vesicles has only been studied sparsely, thus may represent an area that deserves future investigation.

Transport of Glut4 Vesicles along the Cytoskeleton Network Is Mediated by Molecular Motors

Numerous studies have demonstrated that many types of organelle transport in eukaryotic cells are powered by molecular motors along cytoskeleton tracks including the microtubule and actin filaments, particularly when efficient, high-speed transport is

desired [86-90]. Early studies supported the involvement of the cytoskeleton network in insulin-stimulated glucose transport and Glut4 trafficking [91-93]. Consistent with this, recent work has suggested motor proteins of three classes- kinesins, myosins, and dyneins- are involved in different aspects of Glut4 trafficking.

Kinesins represent the largest class of molecular motors that mediate transport along the microtubules [94, 95]. Although early studies suggested a role for kinesin motors in Glut4 trafficking [93], the specific isoforms involved have not been elucidated. Czech and colleagues reported the involvement of Kif5B/Kinesin I in Glut4 trafficking to the plasma membrane, since this isoform appears to be highly expressed in adipocytes and over-expression of dominant-negative mutants of conventional kinesin light chain blocked outward GLUT4 vesicle movements and translocation to the plasma membrane in response to insulin [96]. Work from Olefsky's group suggested another kinesin isoform, Kif3/Kinesin II, is also important for transporting Glut4 vesicles to the plasma membrane [97]. Interestingly, recent studies have suggested that Kif3 associates with the polarity protein complex Par3/Par6/aPKC via a direct interaction through Par3 [98, 99]. It is noteworthy that the latter protein complex has been reported to play an important role in insulin-stimulated glucose transport [100, 101]. Thus, it will be of interest to determine whether the Par3/Par6/aPKC complex may regulate the function of Kif3 in transporting Glut4 vesicles in response to insulin.

Cytoplasmic dynein is a multimeric protein complex comprised of two heavy chains containing microtubule motor activity, and several intermediate, light intermediate, and

light chains [102, 103]. In most if not all cases, dynein-mediated transport involves another multimeric protein complex called dynactin to mediate the cargo-motor interaction [104]. Cytoplasmic dynein mediates movements towards the minus-end of microtubules, and is thus generally thought to function in the recycling of cargoes back to the intracellular compartments such as the recycling endosome or TGN [102, 103]. Alternatively, dynein activity is also important to maintain the organization of intracellular compartments, possibly through the mechanic force generated via attachment to the microtubule network [105-107]. Consistent with these observations, inhibition of dynein activity by brief cytoplasmic acidification of 3T3-L1 adipocytes dispersed perinuclear Glut4 localization and inhibited insulin-stimulated Glut4 translocation to the cell surface [92]. Moreover, microinjection of a dynein antibody in adipocytes inhibited internalization of Glut4 from the plasma membrane, and increased Glut4 levels on the plasma membrane in the basal state [108]. Surprisingly, this antibody did not inhibit insulin-stimulated Glut4 translocation to the plasma membrane [108], as one would predict internalization of Glut4 might be important for its recycling and resorting. In this regard, further studies to establish the exact role of dynein motor and its associated proteins such as the dynactin complex in Glut4 trafficking will be enlightening.

Myosins form a super-family of actin-based motor proteins that consist of 15 distinct sub-families [109]. The conventional myosins were initially found in muscle tissues and to mediate muscle contraction [110]. Although this class of myosins is also found in non-muscle tissue culture cells, their primary function is to regulate cytoskeleton dynamics rather than having a direct role in cargo transport [110]. On the other hand, a large

number of unconventional myosins have been suggested to play an important role in powering vesicle movement on the actin filaments [109, 111]. Among these, Myo1c [112-115] and Myosin 5a [116]have been shown to mediate transport of Glut4 vesicles. The first line of evidence implying a role of Myo1c in Glut4 trafficking came from the fact this motor protein is present in membrane fractions resembling insulin-responsive Glut4 vesicles [112]. Furthermore, disruption of Myo1c function by dominant negative mutants or siRNA-mediated knockdown attenuated insulin-stimulated glucose transport [112, 115]. This role of Myo1c in insulin action may require its binding to PI (4,5)P2 through the C-terminal tail region [114, 117, 118]. Nevertheless, it is not clear how Myo1c may recognize the Glut4 vesicles. In Chapter 3, I will discuss the role of RalA GTPase as well as Calmodulin in bridging Myo1c to its cargo vesicles. Yoshizaki et al. reported that Myosin 5a is an Akt2 substrate that modulates Glut4 vesicle translocation. Insulin stimulates Myosin 5a phosphorylation on Ser1650 via Akt2, and this appears to enhance the ability of Myosin 5a to bind actin filaments [116]. Inhibition of Myosin 5a function leads to blockade of insulin-stimulated glucose transport [116]. However, phosphorylation on Ser1650 on Myosin 5 has been suggested to uncouple this motor from its cargo vesicles [119, 120]. Thus, the exact mechanism underline the involvement of Myosin 5a in insulin action requires further attention.

Tethering/Targeting of Glut4 Vesicles Mediated by the Exocyst Complex

Most, if not all, vesicular transport events require a step called tethering, following the delivery by molecular motors on cytoskeleton tracks but preceding the final fusion with the target membrane [85, 121, 122]. Vesicle tethering is then defined as the physical

interaction between the vesicles and target membrane at some distance and perhaps with some flexibility and reversibility [121]. In this regard, tethering factors are normally protein complexes comprised of multiple long coiled-coil subunits that undergo dynamic assembly cycles [122]. Furthermore, these complexes are able to respond to specific small GTPases localized to unique compartments, suggesting that vesicle tethering also contributes to the specificity of vesicle transport, in the process serving as a targeting mechanism [121, 123-125].

Although a detailed mechanism underling vesicle tethering has not been well characterized, numerous studies have demonstrated this step is an essential and integral component for vesicle transport to various destinations [85, 121, 122]. Consistent with this, multiple vesicle tethering complexes have been identified as regulators of vesicle targeting at specific cellular compartments [122]. These include the COG complex [126], the GRAP complex [127-129], and TRAPP I and II complexes that control tethering at Golgi apparatus [130-132]; the class C VPS proteins that regulate targeting on yeast vacuolar membranes [133-135]; and the exocyst complex that directs exocytic vesicles to the plasma membrane [121, 136]. Importantly, although most of these proteins were first identified in yeast as critical regulators of specific trafficking routes, they display high evolutionary similarity in primary sequences, complex organization, and roles in specific trafficking events [122]. Among these, the only known tethering complex at the plasma membrane is the exocyst, a protein complex that also works to target the Glut4 vesicles to fusion sites on the plasma membrane in adipocytes [137-140]. Intriguingly, recent studies have suggested that docking and the subsequent fusion step might represent the rate-

limiting step in insulin signaling in both cultured and primary adipocytes, thus pointing to an pivotal role of vesicle targeting by the exocyst in this process [141-144].

The exocyst was first discovered by Schekman and Novick through genetic screens to identify temperature-sensitive secretory (*sec*) mutant in budding yeast [145-149]. Six *sec* genes, namely Sec3, Sec5, Sec6, Sec8, Sec10, and Sec15, were found to encode proteins that exist in a complex to target secretory vesicle to the plasma membrane [150-152]. It was later found that the complex contains two additional subunits, Exo70 [151] and Exo84 [153]. Importantly, all of these proteins are conserved in the mammalian exocyst complex, which was purified from rat brain shortly after discovery of the yeast exocyst complex [154, 155], suggesting an evolutionarily conserved role of exocyst in multiple organisms. Most of genes encoding the exocyst protein are found to be essential even in yeast [156], and loss of several of these proteins in higher organisms also leads to embryonic lethality [157-159].

The function and regulation of the exocyst complex has been best studied in budding yeast. Upon disruption of the exocyst function in this model organism, the secretory vesicles continue to form and are delivered to the sites of exocytosis; however, their plasma membrane fusion is blocked [160], as is the formation of the cognate SNARE complex [161]. Furthermore, the exocyst proteins genetically and biochemically interact with the SNARE proteins [162], and their regulators including Lgl [163] and Sec1, the only Sec1/Munc18 family protein that regulates the plasma membrane SNARE complex in yeast [164]. This suggests that the primary function of the exocyst is to bridge the

exocytic vesicles to their target sites at the plasma membrane, and perhaps prepare them for fusion [121]. This notion is supported by studies on the trafficking itinerary of the yeast exocyst proteins themselves. Although all exocyst proteins are highly concentrated in exocytosis sites like the bud tips, they appear to arrive there via different mechanisms- six of the exocyst subunits ride on the exocytic vesicles [165], while Sec3 localizes to the exocyst sites on the plasma membrane independent of vesicle trafficking [166, 167], and Exo70 seems to use both mechanisms for reaching exocyst sites [165].

Consistent with the idea that the exocyst works to bridge vesicles to the plasma membrane, studies in yeast have shown that the subunits of exocyst proteins are anchored by different small GTPases on both membrane compartments (Figure1.3) [168, 169]. In essence, Sec3, and to some extent Exo70, is recruited to the plasma membrane by activated Cdc42 or Rho3, and serve as the landmark for exocytosis sites [168, 170, 171]. On the vesicle end, another activated GTPase Sec4 recruits a sub-complex containing Sec15, Sec10, Sec8, Sec6, Sec5, Exo84, and a pool of Exo70 to the vesicular membranes, via a direct interaction with Sec15 [165, 172]. Additionally, Exo70 appears to interact with phospholipids such as PI (4,5)P2, which may also contribute to the membrane localization of the protein [173, 174]. Thus, the assembly between the vesicle-localized exocyst subunits and those at the plasma membrane tethers vesicle to specific sites marked by the activated Rho GTPases and perhaps phospholipids [121]. Furthermore, this process is somewhat dependent on the integrity of actin cytoskeleton [166], and powered by the molecular motor Myo2 [165, 175-177], which is recruited to the vesicles

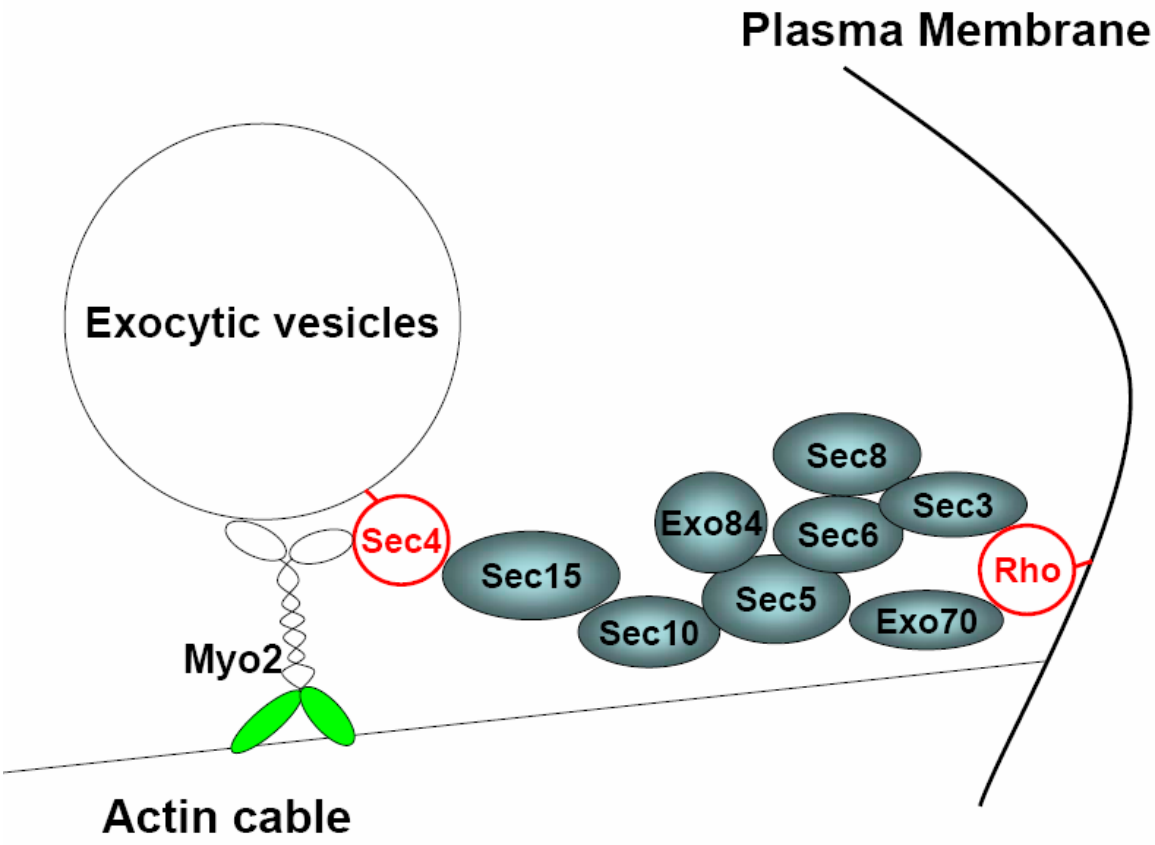

Figure1.3 Potential mechanisms that regulate the function of the exocyst complex during vesicle targeting in yeast The exocyst is anchored on both plasma membranes and the vesicular membranes via interactions with distinct small GTPases that are spatially activated. Part of the exocyst complex including Sec3 and Exo70 is recruited to the plasma membrane by activated Rho family GTPases including Cdc42 and Rho3. On the vesicle end, another activated GTPase Sec4 recruits the exocyst subunits including Sec15, Sec10, Sec5, Sec6, Sec8, Exo84, and a pool of Exo70 to the exocytic vesicles. The assembly of the holo-complex thus bridges the exocytic vesicles to the exocytosis sites on the plasma membrane. Additionally, Sec4 inteacts with the molecular motor Myo2 complex, which mediates the delivery of Sec4-residing vesicles along the actin cables. (Figure modified from Boyd et. al, Ref. 165)

by Sec4 in complex with the myosin light chain Mlc1 [178, 179]. In this way, the exocyst not only contributes to the specificity of vesicle transport to the plasma membrane, via responding to signals that activate specific GTPases; but also enhances the overall velocity of this process; by coordinating the function of the molecular motor Myo2.

In eukaryotic organisms, the exocyst seems to adapt a more specialized function in the endocytic recycling pathway but not the secretory pathway [180-182], despite maintaining an architecture similar to its counterpart in yeast [136, 154, 155]. This was first suggested by genetic studies on Sec5 null flies [157, 158]. Loss of Sec5 caused lethality in files; however, in cultured neurons, only membrane protein insertion on the plasma membrane was impaired, but neurotrasmitter release was intact [157, 158]. Consistent with this, loss of Sec15 in flies causes a targeting defect of photoreceptors that coincides with mis-localization of specific cell adhesion and signaling molecules [183, 184]. This defect is likely due to disruption of endocytic recycling of Notch signaling components [183, 185]. In addition, loss of function of exocyst components Sec5, Sec6, and Sec15 in Drosophila epithelial cells results in DE-Cadherin accumulation in an enlarged recycling endosomal compartment and inhibits DE-Cadherin delivery to the membrane [186]. Finally, a mutant form of the mammalian Sec15 isoform leads to anemia in hemoglobin deficient (hbd) mice, due to impaired endocytic recycling of the transferrin receptor. Taken together, these genetic studies unanimously point to a specific role of the exocyst in regulating plasma membrane targeting of membrane proteins in the endocytic recycling pathway [187-189].

The role of the exocyst in endocytic recycling is also supported by cell biology studies in different cellular contents. The exocyst is required for trafficking to the basal-lateral membrane in epithelial cells, but perhaps not for that to the apical domain [190, 191]. Consistent with this, trafficking of E-cadherin, a basal-lateral membrane-localized protein that undergoes endocytic recycling, is dependent on the function of the exocyst [192-194]. The exocyst also plays an essential role in mammalian cell cycle progression, particularly during the last stage of cytokinesis, the abscission [195-197]. These observations are consistent with the idea that the exocyst mediates vesicle transport to specific domains on the plasma membrane. Furthermore, the exocyst appears to be involved in phagocytosis, a process that also involves concentrated endocytic recycling to the sites of phagosome formation [198].

Despite the importance of the exocyst in vesicle transport to the plasma membrane, it only regulates the targeting of a subset of proteins rather than being an absolute requirement for general trafficking [180-182]. The first indication that the exocyst regulates a unique set of membrane trafficking events came from cell cycle studies. Unlike many proteins involved in vesicle transport during cell cycle, loss of the exocyst function leads to a very specific blockade of abscission, rather than disruption of the early steps in mitosis or cytokinesis that could be caused by global inhibition of membrane transport [196, 197]. This implies that the exocyst only participates in trafficking of proteins that are under tight regulation, particularly since abscission represents the final separation of two dividing cells [199, 200]. This idea is further supported by studies in specialized cell types such as adipocytes, in which both hormone-stimulated, rapid

exocytosis and general trafficking/secretion exist [38, 40]. In this regard, studies have demonstrated that the exocyst is involved in insulin-stimulated Glut4 trafficking to the plasma membrane, but not in recycling of the transferrin receptor or secretion of certain adipokines in adipocytes [138].

The differences in the involvement of the exocyst in certain trafficking processes are at least partly due to the mechanisms underlining the mobilization of the complex [168, 169]. Similar to the yeast complex, the mammalian exocyst interacts with a number of small GTPases in their active states, including TC10 [139, 201], RalA [202-204], Rab11 [185, 205], and Arf6 [206]. Hence, mobilization of the exocyst may require activation of signaling cascades that promote the activity of these GTPases, thus restricting the employment of exocyst in rapid exocytosis that tightly regulated by hormones. Consistent with this hypothesis, I will discuss how the function of the exocyst is regulated via the action of RalA GTPase in the latter part of the thesis.

Plasma Membrane Fusion of Glut4 Vesicles is Mediated by the SNARE Complex and Associated Proteins

Fusion between two membranous cellular compartments such as vesicles and their target membrane organelle, are catalyzed by a protein complex named the SNARE complex [207, 208]. The first indication for the presence of proteins that specialized in catalysis of membrane fusion came from studies aiming to reconstitute Golgi transport *in vitro* [207]. Two cytosolic proteins, the N-ethylmaleimide (NEM)-sensitive fusion protein (NSF) and the soluble NSF attachment proteins (SNAPs), were identified in membrane fusion in

Golgi-derived membrane compartments. Subsequently, membrane receptors for these cytosolic factors were discovered and named as SNAREs for **S**ouble **N**SF **A**ttachment protein **RE**ceptors [207, 208].

Based on their different sub-cellular localization, the SNARE complex can be classified into two categories: the t-SNAREs that localize on the target membrane, and the v-SNAREs that reside on the vesicles [209]. T-SNAREs include members of the Syntaxin family, as well as their binding partners SNAP-25 or SNAP-23 (here SNAP stands for synaptosome-associated protein, but not soluble NSF attachment protein); v-SNAREs are comprised of members of the VAMP proteins [208]. Membrane fusion is catalyzed by the formation of the cognate SNARE complex between the t- and v-SNAREs. This was best illustrated by several biophysics and structural studies, which have shown that cognate v- and t-SNAREs interact through coiled-coil domains to form a very stable four-helix bundle sufficient to overcome the energetic barrier of membrane fusion [210, 211]. Subsequently, the SNARE complex is disassembled through ATP hydrolysis of the ATPase NSF, thereby freeing the SNARE proteins for recycling and the next round of membrane fusion [208, 210, 211].

Syntaxin4 and SNAP-23 are the t-SNARE proteins implicated in Glut4 plasma membrane fusion [212]. Introduction of the cytosolic domain of Syntaxin4 into adipocytes blocked Glut4 translocation to the plasma membrane [213]. Similar inhibitory effects were observed by injection of a blocking antibody against Syntaxin4 [214]. In addition, while Syntaxin4 homozygous knockout mice are not viable, Syntaxin4

heterozygous mice appear to have impaired insulin-stimulated glucose transport in muscle [215]. Consistent with this, SNAP-23, which interacts with Syntaxin4 in adipocytes, has also been implicated in Glut4 vesicle fusion with the plasma membrane after insulin stimulation [216].

Both VAMP2 and VAMP3 have been suggested as potential v-SNAREs that regulate the plasma membrane fusion of Glut4 vesicles [213]. Both VAMP proteins co-localize with Glut4 on intracellular vesicles, and their plasma membrane localization is increased after insulin stimulation [48]. In addition, due to their similarity in the Syntaxin-binding coiled-coil domain in the C-terminus, both proteins are able to form a functional SNARE complex with Syntaxin4 [213, 217]. In this regard, over-expression of the cytosolic domain of either VAMP2 or VAMP3 inhibits Glut4 translocation to the plasma membrane [213]. Nevertheless, VAMP2 and VAMP3 appear to mark different vesicular compartments, probably due to differences in the N-terminus primary sequences of these two proteins [218, 219]. Indeed, membrane compartment ablation studies showed that VAMP3 localizes predominantly to endosomal compartments defined by the transferrin receptor. Although these compartments contain a portion of GLUT4, only a minor fraction of the total population of VAMP3 is found [218, 220, 221]. Furthermore, ablation of the endosomal population enriched for VAMP3 and the transferrin receptor results in a minor effect on insulin-stimulated GLUT4 translocation [220]. Consistent with this idea, mice lacking VAMP3 appear to have normal glucose metabolism [222]. Nevertheless, loss-of-function studies on VAMP2 in animal or cellular models of glucose transport have not been evaluated due to the fact that VAMP2 null mice are not viable [223].

Although the SNARE proteins may represent the minimal requirement for membrane fusion, their *in vivo* functions are tightly regulated by associated proteins in specific trafficking processes, particularly by proteins from the Sec1/Munc18 family [68, 224]. Munc18c of the Sec1/Munc18 family and Synip have been reported to modulate the function of VAMP2-Syntaxin4-SNAP23 complex during plasma membrane fusion of Glut4 vesicles [68]. Sec1 was first identified in yeast as a critical regulator of vesicle fusion on the plasma membrane, and was subsequently shown to bind the assembled SNARE complex [145, 147, 225]. Homologous proteins of Sec1 were found be play an evolutionarily conserved role in mediating vesicle fusion at the plasma membrane [224]. However, nSec1/Munc18a, the neuron-specific isoform of Sec1/Munc18 family was reported to only bind to Syntaxin and compete with VAMPs for this interaction [226]. In this regard, Munc18 isoforms were regarded as negative regulators that prevent the formation of cognate SNARE complex. A similar model was proposed for Munc18c, the ubiquitous isoform of Munc18 family that is also involved in insulin-stimulated Glut4 trafficking[212, 227-230]. Over-expression of full length Munc18c in adipocytes inhibits insulin-stimulated glucose transport [228]. In addition, adipocytes differentiated from Munc18c-null MEFs show increased sensitivity to insulin-stimulated GLUT4 externalization, while the knockout animal is not viable [231]. Nevertheless, it is noteworthy that loss of Munc18c leads to decrease cellular levels of Syntaxin4, an essential component of the SNARE complex, thus it is not entirely clear whether or how loss of Munc18c actually leads to increased formation of the cognate SNARE complex [231]. Furthermore, Munc18c heterozygous mice display significantly decreased insulin

sensitivity in the insulin tolerance test and a >50% reduction in skeletal muscle insulin-stimulated GLUT4 translocation when compared with wild-type mice [232]. This indicates that, in contrast to what was originally proposed, Munc18c may actually serve as a positive regulator of the SNARE complex. Consistent with this idea, a recent study revealed that Munc18a is able to bind the assembled SNARE complex and acts as a stimulatory subunit of its cognate SNARE fusion machinery [233]. Similarly, Munc18c interacts with both the monomeric Syntaxin4 as well as the assembled Syntaxin4-VAMP2-SNAP23 complex [234-236]. In summary, although Munc18c has been domonstrated to play an essential role in facilitating Glut4 vesicle fusion with the plasma membrane, the exact mechanism involved in the process remains to be elucidated.

An additional Syntaxin4 binding protein involved in insulin action is Synip, which was identified using Syntaxin4 as a bait protein in yeast two-hybrid screen [237]. Synip contains a PDZ and EF hand domain at the N-terminus, two tandem coiled-coil domains in the middle, and a WW motif at its C-terminus. Insulin induces a dissociation of the Synip-syntaxin 4 complex. In contrast, the carboxyterminal domain of Synip does not dissociate from syntaxin 4 in response to insulin stimulation and inhibits insulin-stimulated glucose transport [237].

Endocytosis of Glut4 Mediated by Clathrin-dependent Mechanisms

Compared to the exocytosis of Glut4, the endocytosis of the transporter is less well understood. Theoretically, endocytosis of Glut4 is an integral part of the Glut4 trafficking itinerary, particularly for the recycling of the transporter and the re-generation of exocytic

vesicles [238]. Furthermore, decreasing the internalization rate of Glut4 on the plasma membrane may lead to increased glucose uptake, assuming that Glut4 is not required for intracellular delivery of glucose [26].

Although it is widely accepted that insulin stimulation increases the exocytosis rate of Glut4, whether insulin stimulates or inhibits Glut4 endocytosis rate is controversial, perhaps due to the small extent of changes (~ 2 fold regardless of increase or inhibition) exerted by insulin [7, 27, 238]. Nevertheless, several studies suggest that endocytosis of Glut4 is mediated by a clathrin-dependent mechanism [239, 240]. For instance, Glut4 is localized to clathrin-coated pits by immuno-fluorescence and electron microscopy [239, 240]. Also, inhibition of Clathrin function via several methods also prevents Glut4 endocytosis [241, 242].

Like most proteins that undergo clathrin-dependent internalization, the endocytosis of Glut4 is mediated by the large GTPase dynamin [26, 243]. Dynamin contains an amino-terminal GTPase domain, a central pleckstrin homology domain, and a carboxyl-terminal proline-rich region [244]. The function of dynamin in endocytosis was first observed in studies examining the dynamin homolog *shibire* in flies [245]. Temperature-sensitive mutations in *shibire* result in a paralytic phenotype at the non-permissive temperature, due to the absence of synaptic vesicles and an abundance of clathrin-coated pits in the pre-synaptic regions of these flies [246]. Furthermore, these clathrin-coated pits are found to have electron-dense collars around their necks, suggesting that a late stage of endocytosis was inhibited [246]. This defect resulted from altered GTPase activity of

dynamin, as the *shibire* temperature-sensitive mutation occurs near the GTPase domain. Furthermore, experiments employing GTPγS, the non-hydrolysable analogy of GTP, also suggest that dynamin's GTPase activity was necessary for the vesicle scission after invagination of clathrin-coated endocytic vesicles [247, 248]. It has been proposed that GTP hydrolysis by dynamin leads to a conformational change of the protein such that the necks of invaginating vesicles are constricted to the point where membrane scission occurs and free vesicles released [248]. However, it is also possible that the energy derived from GTP hydrolysis causes the elongation of dynamin spirals, resulting in the stretching and eventual scission of membranes on neck of the forming vesicles [247, 249]. Alternatively, dynamin may instead function to recruit downstream effector molecules, including endophilin, which then participate in the formation of released vesicles [243].

Although the detailed mechanisms underlining its function remain to be established, dynamin clearly plays an important role in GLUT4 endocytosis [250-252]. Introducing dominant-negative dynamin mutants or specific peptides to disrupt dynamin function into adipocytes and muscle cells results in a significant inhibition of GLUT4 endocytosis [250-252]. Furthermore, insulin reportedly induces the tyrosine phosphorylation of dynamin [253]. Additionally, insulin may cause accessory proteins to associate with dynamin via its SH3 domain, thus modulating the function of the latter protein [254]. Hence, testing these possibilities will contribute to our understanding of the endocytosis of Glut4.

INSULIN SIGNALING THAT REGULATES GLUT4 TRAFFICKING

Despite much attention, the insulin signaling events leading to Glut4 vesicle exocytosis remain to be fully elucidated (Figure 1.4). Insulin signaling is initiated when the hormone binds to its cell surface receptor, a heterotetrameric protein complex consisting two α and two β subunits [26]. Upon ligand binding to the α subunits, insulin receptor β trans-phosphorylate each other on three tyrosine residues in the activation loop [100]. This results in increased tyrosine kinase activity, which leads to increased autophosphorylation on other tyrosine residues in the juxta-membrane regions and the cytosolic tail, and eventually activation of the receptor [255]. Once activated, the insulin receptor is able to recruit and then phosphorylate a number of substrates, including the insulin receptor substrates (IRS) family proteins, IRS5/DOK4, DOK5, SHC, Gab-1, APS, Cbl, and SIRP family proteins [100, 255]. Most of these substrates, such as IRS proteins or SHC, bind to the juxta-membrane regions of the insulin receptor via an NPXY sequence when the last tyrosine residue is phosphorylated [100, 255]. However, APS binds directly to the activation loop of the receptor [256, 257]. The subsequent phosphorylation on specific tyrosine residues within these substrate proteins creates docking sites for effector or adaptor proteins that contain Src-Homology 2 (SH2) domains, thus transducing and propagating signals from the receptor [100, 255].

Phosphatidylinostol 3-kinase-dependent signaling

Of all the insulin receptor substrates, the best characterized are the IRS proteins [255, 258]. Upon tyrosine phosphorylation, the IRS proteins recruit the p85 regulatory subunits of the PI 3-kinases. This frees the p110 catalytic subunits of the PI 3-kinase and leads to its activation [259, 260]. Activated PI 3-kinase then phosphorylates its lipid substrate phosphatidylinostol 4,5-bisphosphate (PIP2) at the 3 position and generates the lipid product phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which recruits and activates a number of downstream kinases, scaffolding molecules, as well as cytoskeleton proteins [261]. Signals from PI 3-kinase can be terminated by 3' lipid phosphatases such as PTEN [262] or SHIP2 [263], although the latter might not be involved in insulin-stimulated glucose transport in adipocytes [264].

The pivotal role of PI 3-kinase dependent signaling in insulin action has been demonstrated by several independent approaches. Introducing into cells the dominant-negative forms of PI 3-kinase inhibits glucose transport, while overexpression of constitutively active forms can partially mimic insulin action [265, 266]. Pharmacological inhibitors of PI 3-kinase such as wortmannin block insulin-stimulated glucose transport [267]. Targeted ablation of the p85 regulatory subunits of PI 3-kinase in mice results in increase insulin sensitivity, presumably due to loss of inhibition of catalytic subunits [268-270]. Moreover, ablation of the p110 subunits in mice causes insulin resistance and glucose intolerance [271]. Taken together, these data demonstrate an essential role for PI 3-kinase in insulin action.

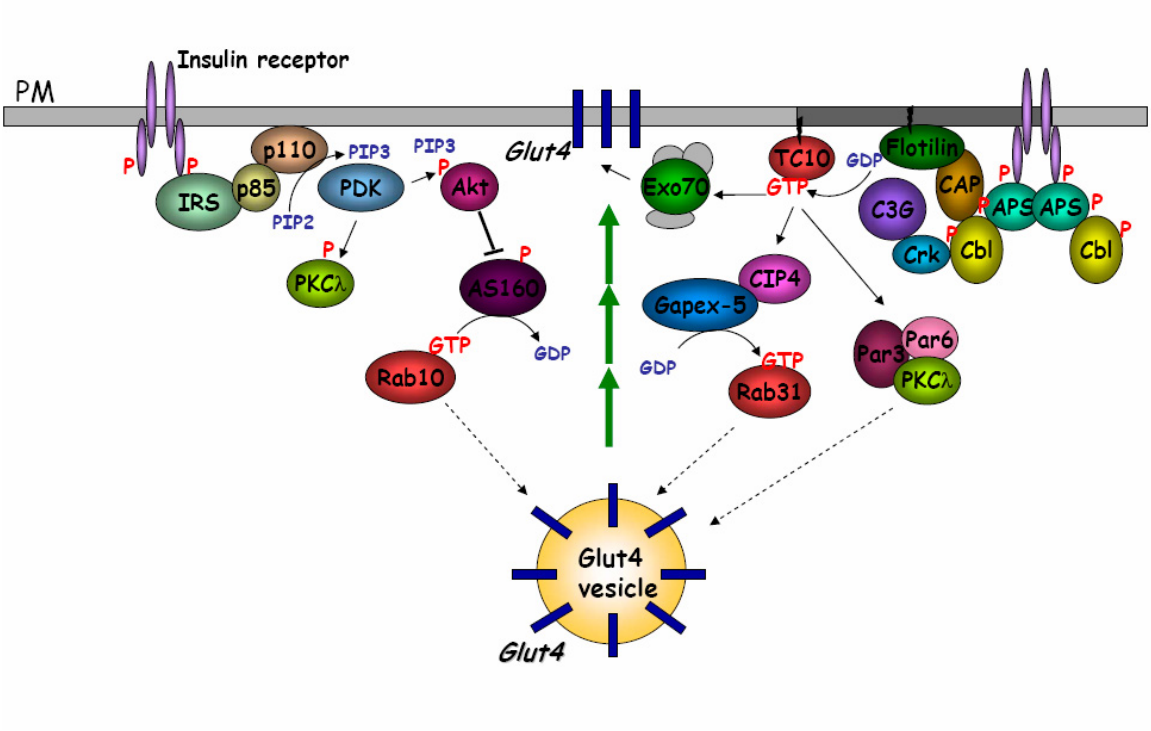

Figure 1.4 Overview of insulin signaling events that regulate Glut4 trafficking Upon activation of the insulin receptor on the cell surface, multiple insulin receptor substrates become phosphorylated, thus initiating several insulin signaling cascades. The PI 3-kinase-dependent signaling pathway eventually leads to the activation of serine/therenine kinase atypical PKCs and Akt. At least one Akt substrate, AS160, has been implicated in Glut4 trafficking, presumably through regulation of its downstream GTPases such as Rab10. The PI 3-kinase-independent pathway eventually results in the activation of a small GTPase named TC10, which then moblizes a number of downstream effectors including the Par3/Par6/aPKC complex, the CIP4/Gapex-5 complex, and the exocyst complex. Among these, Gapex-5 is able to modulate the activity of the Rab31 GTPase, which is impicated in intracellular retention of the Glut4 vesicles. (Figure modified from Chang et al., Ref.5)

Insulin-stimulated PI 3-kinase catalyzes the production of PIP3, which in turn recruits a number of downstream serine/therenine kinases through their pleckstrin homology (PH) domains. One such kinase is PDK1, which in turn phosphorylates additional kinases, including Akt1-3, atypical PKCs, and serum and glucocorticoid-inducible kinases (SGK) [272]. Additionally, activation of Akt kinases requires phosphorylation by mammalian Target of Rapamycin (mTOR) in complex with the adaptor protein rictor [273-275]. While the exact role of atypical PKCs in metabolism remains to be elucidated, multiple studies support a crucial role of Akt kinase in insulin action [276-279]. Remarkably, the two Akt isoforms, Akt1 and Akt2, appear to have differential physiological roles, despite their similar primary sequences [280]. Mice lacking Akt1 are growth retarded, but with less alteration in glucose metabolism [281]. In contrast, Akt2-null mice become insulin resistance and develop diabetes with aging[282]. Knockdown studies using siRNA in cell models like 3T3-L1 confirm the involvement of Akt2, but not Akt1, in insulin-stimulated glucose transport [276]. Nevertheless, the mechanisms underlying this differential involvement of Akt isoforms remain unclear, particularly since overexpression of either isoform can compensate for the defects from loss of the other [280]. Moreover, depletion of both isoforms in adipocytes results in more profound inhibition of insulin action [276], although interpretation of such studies are hampered by the numerous transcriptional targets of Akt that lead to changes in the levels of numerous adipocyte proteins that might play important roles in insulin action (Montminy M., personal communication)

Numerous substrates of Akt kinases have been identified, yet only a few have been shown to play a role in insulin-stimulated glucose transport [68]. Using an antibody that

recognized the phosphorylated consensus motif in Akt substrates, Lienhard and colleagues identified two novel substrates of Akt in adipocytes, AS160 [283] and AS250 [284], which are both phosphorylated by insulin. Interestingly, both proteins possess GTPase-activating-protein (GAP) domains, which may function as negative regulators for small GTPases that work as "molecular switches" during regulated vesicle trafficking [285]. AS160 contains a TBC/Rab GAP domain, which may inactivate Rab family GTPases by enhancing their GTP hydrolysis rate [286, 287]. Overexpression of mutant AS160 that is not able to be phosphorylated by Akt in adipocytes inhibits Glut4 plasma membrane translocation in response to insulin [286, 288]. Moreover, this inhibition is relieved when the GAP activity of AS160 is abolished by mutating a critical arginine into alanine [286]. This suggests that the GAP domain of AS160 plays a negative role in insulin action, and phosphorylation of AS160 by Akt is likely to relieve this negative role of the GAP activity [68, 286]. Conversely, knockdown of AS160 results in increased basal glucose uptake and Glut4 plasma membrane localization, an effect that can be blocked by re-introducing wild type AS160, but not the GAP-deficient AS160 [289]. The role of AS160 in insulin-stimulated glucose uptake appears to be conserved in muscle, and AMPK may mediate its phosphorylation in response to exercise [290-293]. Taken together, these data support an important role of AS160 in insulin action, and this requires a functional GAP domain of AS160 [68]. *In vitro*, the GAP domain of AS160 displays activity towards Rab GTPases including Rab2A, 8A, 10, and 14 [287]. However, the *in vivo* target(s) of AS160 remains to be determined; different Rab proteins including Rab10, Rab8A, and Rab14 have been proposed to work downstream of AS160 to

regulate Glut4 trafficking [294, 295]. Moreover, whether the *in vivo* activity states of these Rab proteins are regulated by AS160 or Akt remain to be determined.

The biochemical properties of AS250, as well as its role in insulin-stimulated glucose transport is much less clear [284]. AS250 contains a predicted GAP domain that resembles RapGAP in the C-terminus [284], yet it is not clear whether this domain has any GAP activity. Additionally, the target GTPases for this putative GAP remain unknown. Knockdown of AS250 leads to a small increase in Glut4 translocation in both basal and insulin-stimulated states [284]. Interestingly, AS250 exists in complex with a novel protein KIAA1219, in a manner that is similar to the TSC1/TSC2 complex [296]. It is noteworthy that the TSC1/TSC2 complex process GAP activity towards Ras family GTPase Rheb, and this activity is negatively regulated by Akt, which phosphorylates TSC2 at multiple sites [296]. Thus, it remains possible that AS250 may be involved in insulin action, via yet to be defined target GTPases.

Although signals stem from the PI 3-kinase dependent cascades are absolutely required for insulin-stimulated glucose transport, substantial evidence suggest that these are not sufficient [101, 297, 298]. Over-expression of an active form of PDK1 leads to activation of Akt and the atypical PKCs, but has little effect on glucose uptake [299]. Furthermore, activation of PI 3-kinase by PDGF or interleukin-4 does not increase glucose transport in adipocytes [297, 298]. Also, incubation of adipocytes with a membrane-permeable analog of PIP3 fails to stimulate glucose uptake in the absence of insulin [300]. Consistent with this, over-expression of activate PI 3-kinase cannot fully mimic insulin

action [266]. Certain naturally occurred mutants of the insulin receptor retain the ability to activate PI 3-kinase signaling, but fail to induce glucose uptake [301]. Taken together, these data suggest the requirement of PI 3-kinase-independent signaling for the full action of insulin.

Phosphatidylinositol 3-Kinase Independent Signaling

Growing evidence suggest that insulin signaling is restricted into specialized cellular compartments, thus achieving maximal efficiency as well as specificity [302]. One such compartment is the lipid raft, specialized regions of the plasma membrane enriched in cholesterol, sphingolipids, lipid-modified signaling proteins, glycolipids, and glycosylphosphatidylinositol (GPI)-anchored proteins [303]. Multiple signaling proteins, including the insulin receptor, have been localized to lipid rafts [304-308]. Insulin receptor initiates a signaling branch by phosphorylation of the proto-oncogene Cbl [309]. This is facilitated by the adaptor protein APS [310]. APS exists as a preformed homodimer, which is then recruited to the activation loop of the activated insulin receptor through binding between phosphorylated tyrosines on the receptor β subunits and the SH2 domain of APS [256, 257]. Upon binding to the receptor, APS is in turn phosphorylated on a tyrosine residue at the C-terminus, creating a docking site for the TKB domain of Cbl [310]. In this way, Cbl is then recruited to the insulin receptor and phosphorylated on 3 tyrosines [310, 311]. This phosphorylation of Cbl is anchored in lipid rafts by the multi-domain adaptor protein CAP [312]. In essence, the C-terminal SH3 domains of CAP interact with Cbl, while the N-terminal SoHo domain of CAP binds Flotillin, a structural protein localized to lipid rafts [312, 313]. CAP is also able to

associate with actin cytoskeleton, via direct binding to vinculin, paxillin, actin and filamin C [313-315]. This cytoskeleton association may also facilitate the organization of signaling platforms, by anchoring signaling molecule into specialized cellular microdomains [308, 316]. This function of CAP is further supported by studies on macrophages or other migratory cells in which CAP is depleted [314, 315, 317].

Upon tyrosine phosphorylation, Cbl interacts with the protein CrkII, an SH2/SH3-containing adapter protein [318]. The SH2 domain of CrkII binds to a phosphorylated tyrosine on Cbl, whereas the SH3 domain of CrkII is constitutively associated with the nucleotide exchange factor C3G [311, 318, 319]. In this way, insulin promotes the translocation of both CrkII and C3G to lipid rafts [319]. Upon its recruitment to lipid rafts, C3G can catalyze the activation of the small G protein TC10 isoforms, which constitutively reside in lipid raft microdomains [319, 320]. Taken together, these studies suggest that insulin initiates a signaling cascade that leads to the activation of the Rho family small GTPase TC10. As will be discussed later, this activation of TC10 is required for glucose transport [5].

SMALL GTPASES THAT CONNECT CELLULAR SIGNALING AND TRANSPORT MACHINERIES

Despite much effort to elucidate the mechanisms by which insulin regulates glucose transport, gaps still exist in our knowledge of how the hormone exerts its action [68, 100, 255]. One essential question is how signaling initiated from the insulin receptor is linked

to transport machineries that modulate trafficking of Glut4 vesicles [68]. Although not completely understood, emerging evidence suggest that members of the Ras small GTPase super-family may fulfill some of this role [100, 321].

Small GTPases exist in eukaryotes from yeast to human and constitute a super-family consisting of more than 100 members [322]. This super-family, named by its founding member Ras, is categorized into at least five sub-families: the Ras, Rho, Rab, Sar1/Arf, and Ran families [322]. These proteins share a similar structure, with a consensus sequence that enables GDP/GTP binding and GTP hydrolysis; a region that interacts with downstream effector proteins; and a C-terminal region that undergoes post-translational lipid modifications, though this region is at the N-terminal of Arf proteins and is absent in Sar1 or Ran family proteins [322, 323].

The most important feature of small GTPases is their ability to adapt to different conformations in the GDP or GTP bound states, which have different affinities for specific effector proteins [324, 325]. This was elegantly revealed by structural studies on multiple members of small GTPases, which were found to contain two highly flexible regions surrounding the γ-phosphate of GTP termed as the switch regions, the switch I region within loop L2 and β_2 and the switch II region within loop L4 and helix α_2. Binding to GTP allows switch I region to adapt to an "active" conformation that enables interactions with downstream effectors, whereas GDP-bound states are unfavorable for these interactions [323, 326, 327]. Furthermore, small GTPases are able to cycle between GTP- or GDP-bound states, a process utilizing their intrinsic GTPase activity and also

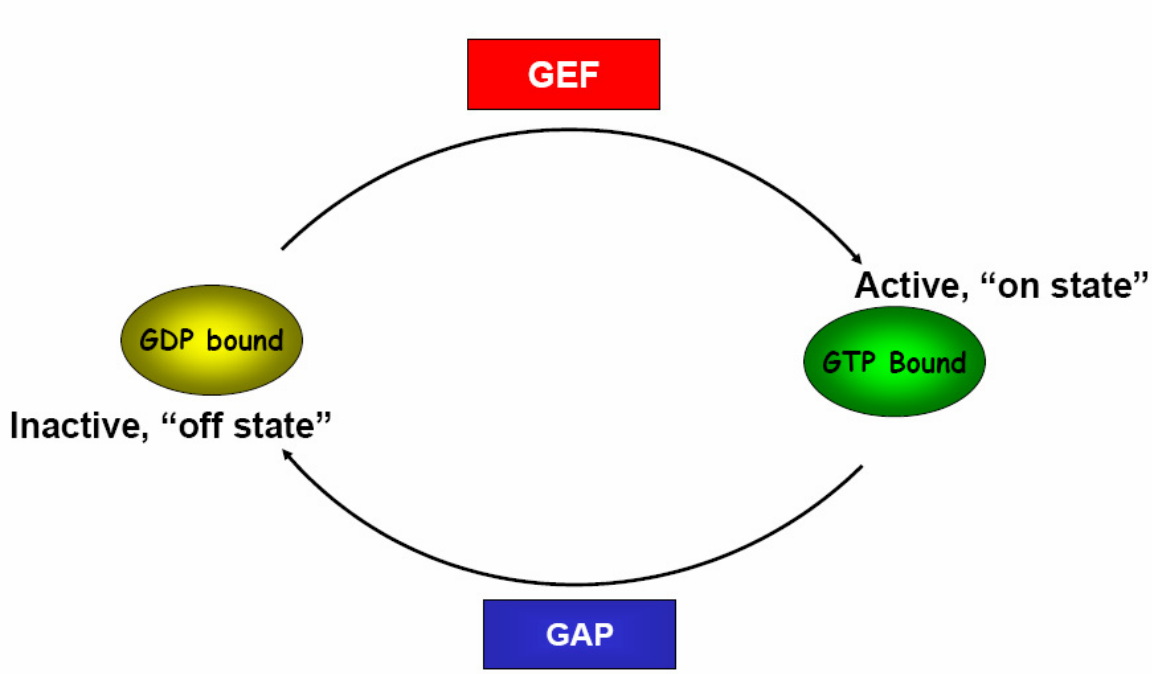

Figure 1.5 The small GTPase cycle Small GTPases can cycle between its GDP bound, inactive state (“off state”) and the GTP bound, active state (“on state”). In the active states, the GTPases are able to interact with their downstream effectors, resulting in their activation or mobilization. The activation of small GTPases is facilitated by guanine nucleotide exchange factors (GEFs); whereas GTPase-activating proteins (GAPs) accelerate the intrinsic GTPase activity of the small GTPases and lead to the inactivation of the GTPases.

facilitated by additional proteins including guanine-nucleotide-exchange factors (GEFs) or GTPase-activating proteins (GAPs) [285]. In this regard, small GTPases are often referred as "molecular switches" for their ability to modulate effector interaction and function via cycling between GTP-bound, active state and GDP-bound inactive state (Figure 1.5) [322].

A growing body of literature has demonstrated that the activity of small GTPases is tightly regulated by signals emerging from membrane-bound receptors [322]. GEF or GAP proteins can be readily regulated by phosphorylation, binding to phospholipids, or association with adaptor proteins, resulting in the activation of specific small GTPases [285]. Upon activation, these small GTPases can then modulate the activity of their effector proteins, including Serine/Threnine kinases, cytoskeleton regulators, transport machineries, and additional adaptor proteins [124, 328, 329]. In this way, the small GTPases represent critical intermediates that integrate upstream signaling from the receptor and mobilize downstream effectors that often directly participate in the transport of vesicle cargos including Glut4 vesicles [100, 321]. In the following paragraphs, I will discuss studies on these small GTPases in the context of insulin signaling and glucose transport (Figure 1.6).

Ras

Although Ras has long been proposed as an important mediator of insulin action, its role in insulin-stimulated glucose transport remains unclear [330]. Earlier studies regarding glucose transport mainly focused on the H-ras isoform, but not K-ras or N-ras [331, 332].

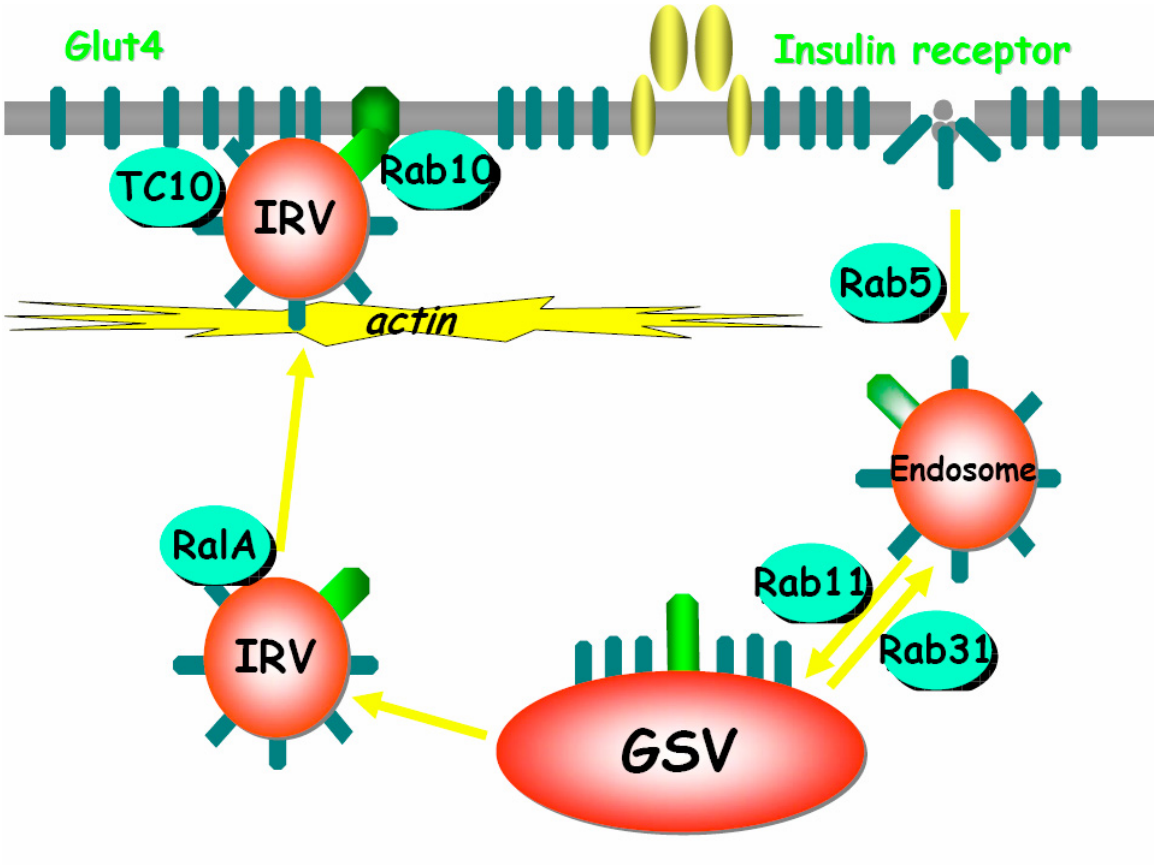

Figure 1.6 Schematic view of the roles of small GTPases in different steps of Glut4 trafficking In the absence of insulin, Glut4 is internalized from the plasma membrane, a step regulated by the GTPase Rab5. Rab11 is thought to regulate the endosome sorting of Glut4 and perhaps the generation of Glut4-Storage Vesicles (GSVs), whereas Rab31 has been proposed to regulate the retention of Glut4 in endosomes. RalA is associated with Glut4 vesicles and may coordinate the movement of these vesicles along the actin cytoskeleton as well as their docking on the plasma membrane. TC10 is activated on the plasma membrane and directly participate in the targeting of Glut4 vesicles to their exocytosis sites. Rab10 may be involved in a distal step in Glut4 exocytosis such as priming or membrane fusion by the SNARE complex and their associated proteins.

The activation of Ras by insulin initiates a series of phosphorylation events resulting in a potent activation of mitogen-activated protein kinases (MAPK) by its upstream kinase MEK [330]. Nevertheless, studies have found that this activation of MAPK only contributes to the mitogenic actions of insulin, with little impact on the metabolic actions of the hormone [333, 334]. Microinjection of activated Ras into adipocytes enhances glucose uptake mainly via up-regulation of Glut1, but has little effect on the insulin-responsive glucose transporter Glut4 [331, 332]. Also, expression of dominant negative Ras in adipocytes has little effect on glucose transport [335]. However, overexpression of active Ras in adipose tissue improves insulin sensitivity of this tissue, in which Glut4 is much more abundant and accounts for most of the glucose uptake [336]. It is noteworthy that Ras is also able to activate PI 3-kinase signaling via a direct interaction with the p85 regulatory subunit of PI 3-kinase [337]. Additionally, Ras activation may lead to enhanced activity of other small GTPases including Ral and Rab5, via direct binding to the RalGEFs [338] or the Rab5 GEF Rin1 [339], respectively. In this regard, Ras effector domain mutants that selectively activate one branch of the downstream signaling [340, 341], as well as siRNAs that deplete Ras isoforms, may be useful tools to further address its role in insulin action.

Ral

Ral GTPases (RalA and RalB) constitute a branch of the Ras sub-family in the small GTPase super-family [342]. Studies on Ral GTPases presented first examples of crosstalk between small GTPases, as a number of RalGEFs contain Ras-binding domains and are under the regulation of activated Ras [338, 343]. However, other signals independent of

Ras activation, including the PI 3-kinase pathway and calcium signaling, have also been implicated in the activation of RalA [342, 344]. Nevertheless, the mechanisms underlying Ral activation remain uncertain. Particularly, as RalGEFs contain a variety of regulatory domains that respond to different upstream signals, the exact role of specific GEF proteins in certain physiological contexts for the most part remain uncertain [345, 346]. On the other hand, no RalGAP protein has been identified, although GAP activity towards RalA has been reported [347].

The fact that Ral is a downstream target of Ras has lead to the idea that Ral may contribute to the oncogenic activity of Ras [342]. This idea was supported by morphological studies in fly eyes using Ras mutants that only activate a branch of downstream signaling including Ral activation [348]. However, other studies have shown that active Ral failed to transform NIH 3T3 cells in a focus-forming assay [340]. Nevertheless, a number of subsequent reports have shown that activation of Ral proteins, as well as RalGEFs, are crucial to cellular transformation [340, 349, 350], particularly in cells derived from human origin [351, 352]. Interestingly, it was recently proposed that RalA and RalB may play different roles in this process, with RalA important for proliferation of tumor cells and RalB important for the survival of these cells [351, 353-355].

Although the differences in cellular function of RalA and RalB are not completely understood, the variations in the N- and C-terminus regions may contribute to this diversity [352]. RalA reportedly interacts with PLD through the N-terminus of the former

protein, a region that is absent in RalB [356]. Furthermore, RalA has been shown to reside both on the plasma membrane and but mostly on the intracellular vesicles, where as RalB is primarily localized to the former compartment [357]. Consistent with this, RalA has been found in a variety of vesicular structures in different tissues, including synaptic vesicles, platelet granules, as well as vesicles enriched with the water channel Aquaporin [358-361]. Furthermore, this vesicular localization has led to the hypothesis that RalA is directly involved in vesicle transport, although this function appears to be unusual for GTPases in the Ras sub-family [342].

The role of RalA in vesicle transport is best supported by the identification of the exocyst as a direct effector complex of the protein [202-204]. Both RalA and RalB interact with two subunits of the mammalian exocyst, Sec5 and Exo84 [202-204], and RalA has higher affinity than does RalB [357]. Furthermore, depletion of RalA disrupts the establishment of cell polarity; a process that also involves the exocyst function [203]. Nevertheless, the exact mechanism by which RalA regulates exocyst function remains uncertain, as is the role of these proteins in regulation of vesicle transport in physiological scenarios including insulin-stimulated glucose transport.

Rho/Cdc42/Rac

The Rho family GTPases including Rho, Rac, and Cdc42 were well characterized for their role in the regulation of cytoskeleton rearrangement, a process thought to be important for various vesicle transport events [328, 329]. However, whether insulin

actively regulates cytoskeleton rearrangement in target tissues remains controversial [91, 100], particularly due to inconsistent results obtained regarding the insulin-induced activation of these Rho GTPases in insulin-responsive cells [91]. Nevertheless, it remains possible that the activities of these Rho GTPases are required for insulin-stimulated glucose transport. Consistent with this, Clostridia toxins that inactivate Rho GTPases and depolymerize the actin cytoskeleton have been shown to inhibit glucose transport in both the basal and insulin-stimulated states in 3T3-L1 adipocytes [362]. However, studies on glucose transport using dominant negative mutants of Rho GTPases are inconsistent [319, 363-365]. It should be noted that expression levels of dominant negative GTPases need to be tightly controlled to achieve desired biological effects while avoiding non-specific toxicity. In this regard, loss of function studies at cellular level using siRNA-mediated knockdown or gene ablation in animal models are of interest to delineate the function of these Rho GTPases in insulin-stimulated glucose transport.

TC10

Although both isoforms of TC10 (TC10 α and TC10β) belong to the Rho GTPase sub-family and share high homology to Cdc42 [320], several lines of evidence suggest that this GTPase may be an atypical member of Rho family GTPases. First, unlike another Rho GTPases such as Rho, Rac, and Cdc42 that undergo geranylgeranylation [322, 329], TC10 undergoes tandem palmitoylation and farnesylation at the C-terminal hyper-variable region [366, 367]. This may at least partially explain the differences in subcellular localization between TC10 and other Rho GTPases, particularly Cdc42 [366, 367]. Furthermore, many Cdc42 GEF proteins are unable to activate TC10, suggesting

the activity of these two GTPases are differentially regulated [368]. In addition, unlike Cdc42, TC10 does not exist in lower organisms like yeast, and human TC10 fails to rescue the defects in a *cdc42* mutant like human Cdc42 does [369]. Taken together, these data suggest that TC10 may carry out distinct biological functions that are uniquely regulated in different cellular contexts.

Several lines of evidence suggest that TC10 plays a pivotal role in insulin-stimulated glucose transport [5]. Both TC10 isoforms are enriched in insulin responsive tissues including muscle and fat, and rapidly activated by insulin stimulation [319, 320]. Nevertheless, it appears that only the alpha isoform of TC10 is critically involved in insulin action, as expression of dominant negative TC10α but not TC10β inhibits insulin-stimulated glucose uptake and Glut4 translocation to the plasma membrane [319, 320]. Consistent with this, depletion of TC10α by siRNA-mediated knockdown inhibits insulin-stimulated glucose transport [370]. Furthermore, several studies have identified TC10 effectors that also participate in the metabolic actions of insulin, including the adaptor protein CIP4, Par6 in the Par3/Par6/aPKC complex, and the Exo70 subunit of the exocyst complex [5]. These interactions thus enable insulin to regulate diverse cellular processes via the activation of TC10.

The multi-domain adaptor protein CIP4 contains an N-terminal FCH domain, two central coiled-coil domains, and a C-terminal SH3 domain [371]. Through its SH3 domain, CIP4 is able to associate with a multi-functional protein called Gapex-5 via the PxxP motif of the latter protein [372]. Gapex-5 contains a Ras GAP domain at the N-terminus, a

VPS9/Rab5 GEF domain at the C-terminus, as well as several PxxP motifs in the middle region [372]. The GAP domain of Gapex-5 processes activity towards Ras as well as TC10, while the GEF domain of Gapex-5 enhances the activity of the Rab5 sub-family GTPases including Rab31 [372]. In the basal state, Gapex-5 is primary intracellular and maintains intracellular activity of Rab31 at a high level. Upon insulin stimulation, TC10 recruits CIP4/Gapex-5 complex to the plasma membrane, thus decreasing the activity of Rab31 [372]. As will be discussed later, Rab31 is localized to TGN [373]and appears to prevent Glut4 translocation to the plasma membrane by retaining the transporter in a process of futile cycling [372]. Additionally, recruitment of CIP4/Gapex-5 complex to the plasma membrane by TC10 can then activate Rab5 at the plasma membrane (Lodhi et al, unpublished). Taken together, the CIP4/Gapex-5 branch of TC10 effectors represents a mechanism of compartmentalized activation of GTPases and cross-talk between different GTPases.

Another branch of TC10 effectors is the adaptor protein Par6 of the Par3/Par6/aPKC complex [374, 375]. The atypical PKCs have been implicated in insulin-stimulated glucose transport [376, 377], as has Par3 and Par6 [374]. Active TC10 recruits aPKC to the plasma membrane of adipocytes via association with Par6 and Par3 [374]. It is noteworthy that the activity of atypical PKCs may be under the control of PI 3-kinase signaling [101]. Additionally, as discussed above, the Par3/Par6/aPKC complex may regulate the function of the microtubule motor KIF3/kinesin II [98, 99]. Thus, this branch of TC10 signaling may represent a convergent point with PI 3-kinase signaling, and/or with molecular motor functions.

Activated TC10 also interacts with Exo70, a subunit of the exocyst complex [139, 201]. This interaction recruits Exo70 and at least some of the exocyst subunits to the plasma membrane, a process involved in the docking of the Glut4 vesicles to the plasma membrane [139, 140]. Hence, insulin-induced activation of TC10 may mark the sites of exocyst assembly and direct the docking and eventually fusion of Glut4 vesicles. Regulation of the exocyst by TC10 is also involved in neurite outgrowth [201], as well as cell motility [378]. GTP hydrolysis of TC10 is also required for the fusion of vesicles with the plasma membrane, suggesting that disassembly of the exocyst via TC10 inactivation is also an integral step of vesicle transport [378].

ADP-ribosylation Factors (Arfs)

Although ARF proteins were originally named for their ability to function as cofactors for cholera-toxin-catalyzed ADP-ribosylation of the α-subunit of heterotrimeric G proteins Gs, they have now been shown to regulate membrane trafficking pathways in a variety of processes [379]. Arf6 represents the best characterized isoforms of the Arf GTPase family [379]; however, its involvement in insulin-stimulated glucose transport is controversial [82, 380-384], as is the mechanism by which insulin may regulate its activity [381, 383-385]. This is further complicated by two recent studies suggesting that activation of Arf6 by its GEF protein cytohesin regulates upstream signaling directly emerged from the insulin receptor [386, 387]. On the other hand, the effectors of Arf proteins have been shown to regulate different steps in Glut4 trafficking.

Several studies have suggested a requirement for the Golgi-localized γ-ear-containing Arf-binding proteins (GGA) of coat adaptor proteins in the biosynthetic sorting of insulin-responsive Glut4 vesicles [30, 78, 79]. GGA proteins are able to associate with Arf proteins, clathrin, and the cytosolic tails of intracellular transport receptors, thus mediating formation of Clathrin-coated carriers for specific cargo proteins [30, 76, 77]. Expression of a dominant-interfering GGA mutant inhibited insulin-stimulated GLUT4 translocation and GST–GGA fusion proteins were found to bind to GLUT4-containing transport vesicles but did not directly bind to GLUT4 itself [78, 79]. Moreover, siRNA-mediated knockdown of GGA before GLUT4 expression completely prevented insulin-stimulated translocation of newly synthesized Glut4 [78]. Additionally, GGA's role in Glut4 vesicle sorting may depend on the peripheral Golgi protein Golgin-160 [388]. Taken together, these data indicate that GGA may play a role in assemble clathrin-coated carrier vesicles for Glut4.

Another protein that may mediate Clathrin coat assembly during Glut4 trafficking is ACAP1, a GTPase-activating protein of Arf6 [389]. Interestingly, ACAP1 also functions as an Arf6 effector to interact with both clathrin coats and cargo proteins including TfR [390], β-1 integrin [391], as well as Glut4 [82]. Indeed, siRNA-mediated depletion of ACAP1 or Arf6 inhibits insulin-stimulated glucose transport [82]; consistent with the idea that Arf GTPases regulate coat assembly via adaptor protein binding [379]. Additionally, specific phospholipids such as PI (4,5)P2 may also play a role in membrane recruitment of the adaptor proteins for coat assembly [76, 77]. This idea coincides with

the report that phospholipase D, an Arf6 effector that increases the levels of phosphatidic acid and eventually PI (4,5)P2, may be involved in Glut4 trafficking [392-394].

Rab family proteins

The human genome contains almost 70 Rabs and Rab-like proteins, which constitute the largest sub-family of Ras super-family GTPases [395, 396]. These Rab GTPases are localized and activated at distinct membrane-bound compartments and regulate various aspects of vesicle transport [123, 177, 396]. Consistent with this notion, several Rab proteins have been suggested to participate in distinct steps in insulin-stimulated Glut4 trafficking [68, 321].

First line of evidence suggesting the involvement of Rab GTPases in Glut4 trafficking came from localization studies. Multiple Rab proteins, including Rab4, Rab5, Rab10, and Rab11 have been reported to co-purify with Glut4-enriched vesicular membranes [48, 58, 287, 397]. Among these GTPases, Rab4 and 11 have been implicated in regulating endocytic recycling of membrane proteins to the plasma membrane in various systems [123, 395]. Moreover, the plasma membrane localization of both GTPases in adipocytes or cardiomyocytes is increased by insulin stimulation [220, 398, 399]. Over-expression of the wild-type and mutant form of Rab4 has been shown to block insulin-stimulated glucose transport [400-402]. Furthermore, Rabip4, a FYVE domain containing protein that interacts with both Rab4 and 5, has been implicated in Glut4 trafficking, though the precise role of the protein in this process remains to be determined [403, 404]. The requirement of Rab11 in insulin action remains controversial, due to inconsistent results

obtained using dominant negative form of the GTPase [380, 405]. However, it has been reported that Rab11 is required for the sorting of GLUT4 from endosomes to the specialized compartment, and thus for the insulin-induced translocation to the cell surface [406]. This suggests the importance of the general endosomal pathway in the specialized trafficking of GLUT4 that is regulated by insulin signaling [406]. Furthermore, Rab11 may also participate in the action of a 72 kD inostiol polyphosphate 5-phosphatase, which has been implicated in insulin stimulated PI(3)P production and plasma translocation of Glut4 [405]. The GTPase Rab10 has recently been implicated in Glut4 trafficking, as a downstream target of Akt substrate AS160 [295]. Rab10 has been shown to regulate trafficking to the basal-lateral membrane in MDCK cells, possible via the endocytic compartments [407, 408]. However, the exact role of Rab10, as well as its effector proteins, in insulin-stimulated glucose transport remains to be determined.

Besides Rab11, the intracellular dynamics of Glut4 vesicles may also be controlled by another GTPase Rab31, a Rab5 subfamily GTPase implicated in trans-Golgi network (TGN)-to-endosome trafficking [373]. Rab31 is a downstream target of Gapex-5, via the VPS9 domain of the latter protein [372]. Overexpression of Rab31 blocks insulin-stimulated Glut4 translocation, whereas knockdown of Rab31 potentiates insulin-stimulated Glut4 translocation and glucose uptake. Furthermore, insulin reduces Rab31 activity by recruiting Gapex-5 to the plasma membrane, thus permitting Glut4 vesicles to translocate to the cell surface [372]. In this regard, identification of Rab31 effectors involved in insulin-stimulated glucose transport will be of great interest.

Rab5 is a multi-functional GTPase that regulates the endocytosis of proteins from the cell surface, homotypic fusion of early endosomes, formation of clathrin-coated endocytic vesicles, motility of early endosome on microtubules, and turnover of phospholipids [395]. Numerous downstream effectors of Rab5 have been identified, as key machineries that directly regulate specific steps in endocytic trafficking [409]. Although the role of most Rab5 effectors has not been tested in Glut4 trafficking, insulin stimulation appears to modulate both the localization and the activity of the GTPase [108, 410]. Micro-injection of an anti-Rab5 antibody blocked internalization of plasma membrane localized Glut4; suggesting Rab5 regulates the endocytosis of Glut4 [108]. On the other hand, APPL1, a Rab5-interacting protein that also binds to the adiponectin receptor as well as Akt kinase [411], has recently been reported to regulate the exocytosis of Glut4 [411, 412]. Furthermore, Rab5 is able to regulate phosphoinositide turnover by directly interacting with PI 5- and PI 4-phosphatases and stimulating their activity both at the endocytic compartments as well as the plasma membrane[413], a process potentially important for insulin-stimulated Glut4 trafficking [414, 415]. Hence, the exact role of Rab5 in glucose transport is only being unveiled and future studies on the functions of this GTPase in insulin action are of high interest.

SUMMARY

After almost twenty years since the Glut4 transporter was first cloned, our knowledge of the mechanisms by which insulin regulates its function is still limited. Although there is substantial evidence that insulin activates at least two parallel signaling pathways that

govern the trafficking of Glut4, the detailed mechanistic actions of the signaling cascades are not completely understood [100, 255]. On the other hand, among the many transport machineries implicated in Glut4 trafficking, few have been shown to be direct targets of insulin [26, 68]. In this regard, key to this question is to identify signaling intermediates that directly modulates the function of transport machineries [100, 321]. Delineation of the actions of these intermediate proteins, as well as the means by which insulin regulates the activity of these proteins, is likely to yield much information on how insulin is able to exert its unique action in stimulating glucose transport. Growing evidence suggests that members of the small GTPase family may represent such signaling intermediates. This thesis focuses on the action of the GTPase RalA, which directly interacts with the vesicle-tethering complex known as the exocyst. In Chapter 2, I will discuss the role of RalA and the exocyst in regulating a subset of endocytic recycling pathway particularly during a process called cytokinesis. In chapter 3, I will present evidence that RalA regulates insulin-stimulated endocytic recycling of Glut4 via the interactions between the exocyst and the molecular motor Myo1c. In Chapter 4, I focus on delineating the molecular architecture of the exocyst under the regulation of RalA. In Chapter 5, I will briefly discuss the potential mechanism of RalA activation, as well as a negative feedback loop that uncouples RalA from the exocyst. Taken together, these studies suggest RalA serves as an important intermediate that links upstream signaling to the exocyst during insulin-stimulated Glut4 trafficking and a few other processes.

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Chapter 2

RalA-Exocyst Dependent Recycling Endosome Trafficking is Required for the Completion of Cytokinesis.

Introduction

Cytokinesis is a crucial process in which the cytoplasmic constituents of the mother cell are divided into two identical daughter cells, ensuring the fidelity of cell division. Cytokinesis proceeds via distinct steps, including assembly of the actomyosin contractile ring, formation of the ingressing cleavage furrow, and cell cleavage or abscission [1]. Membrane trafficking is important for all steps of cytokinesis and is directly required for sealing off the abscission site where cells undergo the final separation [2]. Recycling endosome-derived vesicle trafficking plays an essential role in the terminal stages of cytokinesis, possibly under the control of the centrosome [3]. However, the mechanisms underlying this polarized delivery of vesicles from the recycling endosomes during abscission remain poorly understood.

The exocyst is an evolutionarily conserved vesicle tethering complex, comprised by eight subunits including Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 [4].

This complex has been proposed to mediate the initial recognition between the exocytic vesicles and the target membrane, thereby contributing to the specificity and efficiency of certain vesicular transport processes [5]. Recent insights into structure of the exocyst have shed light on the architecture and function of this complex, suggesting that the exocyst assembles into an overall rod-like structure, in the process bridging the vesicles to their target membrane [5, 6]. Consistent with this notion, the exocyst has been found to concentrate on "hotspots" on the plasma membrane where exocytosis actively takes place, and has been implicated in different types of membrane trafficking including polarized growth in yeast, neurite growth in the nervous system, glucose transport in fat cells and basal-lateral trafficking in epithelial cells [4]. Interestingly, in *S. cerevisiae* and *S. pombe*, the exocyst complex localizes to the cleavage furrow and is essential for membrane delivery during cytokinesis [7-9]. However, the role of the exocyst in cytokinesis is poorly understood in mammalian cells, in which the exocyst has a more specialized yet complex function.

Ral GTPases (RalA and RalB) have been the focus of special attention for their roles in regulating exocyst function in eukaryotic cells. Upon activation, Ral can bind two exocyst subunits, Sec5 and Exo84. RalA has higher affinity for these proteins than does RalB [10]. While the molecular mechanisms remain elusive, this unique interaction pattern may enable RalA to regulate the assembly of the exocyst during vesicle targeting, as Sec5 and Exo84 seem to have different cellular localizations [11] . Nevertheless, although RalA has been reported to regulate exocytosis in several scenarios, the generalized function of this ubiquitously expressed small GTPase in vesicle trafficking

remains largely unknown. Importantly, RalA has also been implicated in signaling pathways controlling cell cycle progress, cell morphology, and oncogenic transformation [12]. A recent report highlights the oncogenic function of RalA, but not RalB, and the involvement of the exocyst in RalA-induced cellular transformation [13]. However, it is not clear whether RalA-mediated vesicular trafficking is directly involved in cell cycle progression.

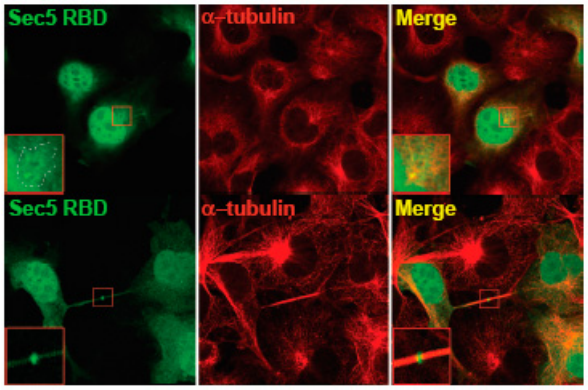
Here we present data suggesting a critical role for RalA and the exocyst in targeting RE-derived trafficking during the completion of cytokinesis in mammalian cells. RalA is RE-associated and re-localizes to the cleavage furrow and later the abscission site. The exocyst, through a spatially and temporally regulated association with key cytokinetic structures, regulates the targeting of RalA-containing vesicles from RE. Disruption of this process leads to late stage cytokinesis failure.

Results

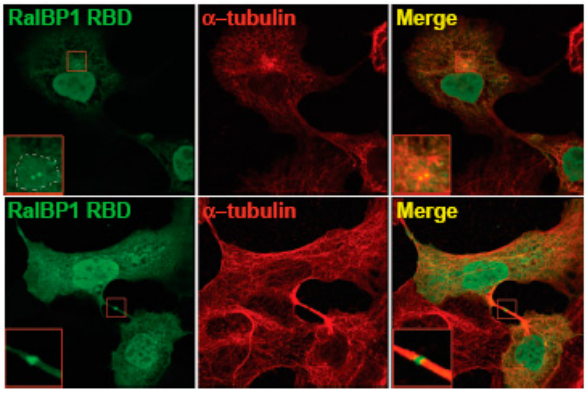
Active Ral GTPase is localized on the pericentrosomal membrane and at the centrosome. To explore the function of the Ral GTPase, we generated an eYFP-tagged Ral-binding domain (RBD) of Sec5 to probe the localization of active Ral *in vivo*. In contrast to previous studies in polarized MDCK cells [14, 15], the fluorescent fusion protein mainly decorated the perinuclear region in the cytoplasm of quiescent Cos-1 cells rather than the plasma membrane (Figuew 2.1A, upper panel). We noticed that this

Figure 2.1 Active RalA is localized on the pericentrosomal membrane and at the centrosome. (A-B) Cos-1 cells expressing eYFP-tagged Sec5 RBD (A), RalBP1 RBD (B) were stained using α-tubulin (red) antibody. RBDs (green) localize around and at the centrosome in interphase cells (upper panel), and the abscission site in cytokinetic cells (lower panel). **(C)** eYFP-Sec5 RBD T11A shows a disorganized punctate staining when expressed in COS-1 cells. **(D)** Affinity of the RBD constructs with RalA. Cos-1 cells were transfected with RalA and indicated eYFP-constructs, lysed, and subjected to immunoprecipitation using an anti-FLAG antibody. IP and lysates were blotted with FLAG or GFP antibody after SDS-PAGE. **(E)** eGFP-RalA localizes around and at the centrosome in Cos-1 cells as visualized by pericentrin (blue) and α-tubulin (red) staining.

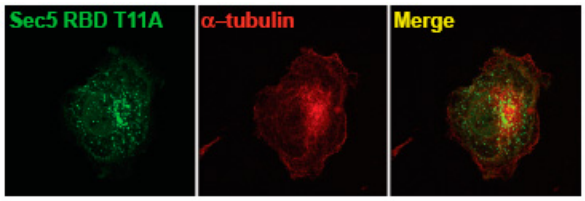
A


Sec5 RBD
α–tubulin
Merge
Sec5 RBD
α–tubulin
Merge

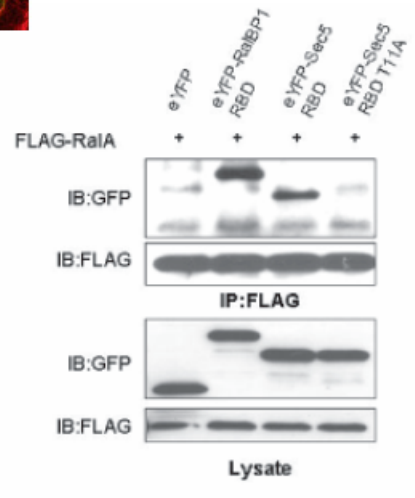
B


RalBP1 RBD
α–tubulin
Merge
RalBP1 RBD
α–tubulin
Merge

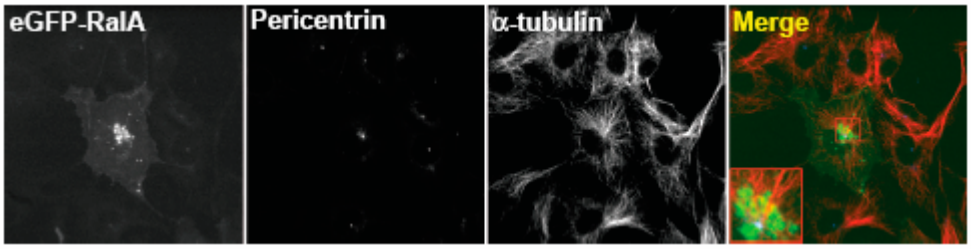
C


Sec5 RBD T11A
α–tubulin
Merge

D


eYFP
eYFP-RalBP1 RBD
eYFP-Sec5 RBD
eYFP-Sec5 RBD T11A
FLAG-RalA
+ + + +
IB:GFP
IB:FLAG
IP:FLAG
IB:GFP
IB:FLAG
Lysate

E


eGFP-RalA
Pericentrin
α-tubulin
Merge

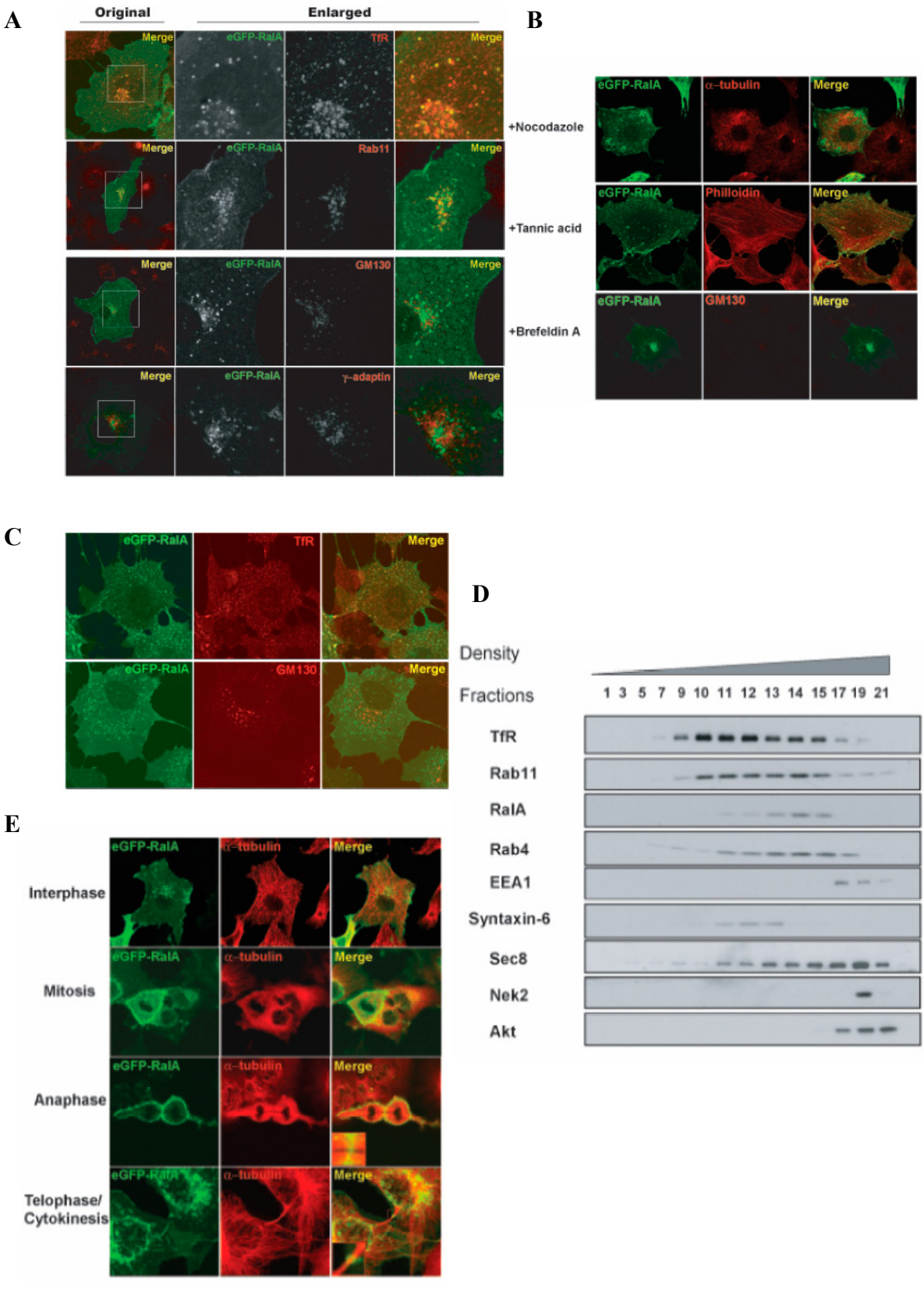
fluorescent protein also localized to the nucleus, as GFP is known to non-specifically diffuse throughout the cytoplasm and nucleus. The nuclear-localized fluorescent RBDs, however, may be isolated from Ral in the cytoplasm due to the presence of the nuclear envelope, and thus cannot function as probes for activated Ral. Interestingly, in ~20% of the transfected cells, Sec5 RBD decorated one or two bright dots near the nucleus. Co-staining with alpha-tubulin reveals that Sec5 RBD localized to the centrosome in interphase cells, as well as the centrosome-related abscission site in cells undergoing cytokinesis (Figure 2.1A, lower panel). The same results were obtained with the RBD from RalBP1, another Ral-interacting protein (Figure 2.1B). Nevertheless, Sec5 RBD T11A showed a distinct localization and did not concentrate at the peri-centrosomal region (Figure 2.1C), due to its substantially lower affinity for active Ral (Figure 2.1D). Taken together, the data indicate that endogenous Ral can be activated at the centrosomal and pericentrosomal membranes.

Several recent studies have implicated Ral GTPase in vesicle trafficking [12], although the exact mechanism remains to be elucidated. We found that ectopically expressed RalA also co-localized with eYFP-RBDs in the same pericentrosomal tubule-vesicular structures, suggesting that a large fraction of active RalA associates with intracellular membranes in addition to the plasma membrane (data not shown). Indeed, we observed that wild type RalA localized to the vesicular structures surrounding the centrosome/microtubule organization center (MTOC) and associated with the microtubule filament (Figure 2.1E).

RalA is a Recycling endosome (RE)-localized GTPase that re-localizes during cell division. Despite the general notion that RalA can localize to the exocytotic vesicles in some specialized cells such as neurons and platelets [12], the exact subcellular localization of this widely expressed GTPase is unclear. The pericentrosomal localization of RalA closely resembles the Golgi apparatus or the recycling endosome, two organelles often organized around the MTOC. Consistent with a study in polarized MDCK cells by Feig and colleagues [10], we observed that RalA co-localized with the recycling endosome markers Transferrin Receptor (TfR) and partially with Rab11 in non-polarized Cos-1 cells, but poorly with GM130 or γ-adaptin, which decorate the *cis* and *trans* Golgi membrane, respectively (Figure 2.2A). In addition, disruption of the microtubule network by nocodazole or inhibition of vesicle flow by tannic acid [16] abolished the pericentrosomal localization of RalA, whereas disassembly of Golgi by Brefeldin A had little effect (Figure 2.2B).

Nocodazole is able to disrupt the peri-centrosomal localization of both recycling endosomes and the Golgi apparatus, separating these organelles into geographically distinct structures. We used this approach to further define the localization of RalA. As expected, RalA precisely co-localized with TfR in peripheral vesicular structures, but not with GM130 (Figure 2.2C), suggesting a preferred localization of RalA to the recycling endosome but not to Golgi. Furthermore, endogenous RalA partially overlapped with the TfR and Rab11, but not with the early endosome marker EEA1, the Golgi protein Syntaxin-6, or the cytosolic protein Akt in a linear opti-prep gradient (Figure 2.2D). Notably, RalA also co-fractionated with Rab4, another GTPase known to regulate

Figure 2.2 RalA is a recycling endosome (RE)-localized GTPase that re-localizes during cell division. (A) RalA co-localizes with TfR and Rab11, but not with γ-adaptin or GM130. Cos-1 cells transfected with eGFP-RalA WT were stained with antibodies against indicated proteins (red). **(B)** Nocodazole or tannic acid, but not BFA treatment, dramatically changes the localization of RalA. Cos-1 cells transfected with eGFP-RalA WT were treated with 33μM nocodazole for 2 hours (upper panel), or 0.5% tannic acid for 10 minutes (middle panel), or 5 ug/ml Brefeldin A (BFA) for 2 hours (lower panel) and stained with antibodies against indicated protein. **(C)**Nocodazole treatment causes RalA to localize to punctuate structures that co-localizes with TfR, but not GM130. **(D)** Cellular fractionation profile of RalA. Cos-1 cells were homogenized and the post nuclear supernatant was separated using a linear 10-20-30% opti-prep gradient. Equal volume of each fraction was loaded on a 4-20% SDS-PAGE gel. Distribution of different proteins was determined by Western blot. **(E)** Cell cycle-dependent re-localization of RalA. Cos-1 cells expressing eGFP-RalA WT were stained with α-tubulin (red) antibody to determine the stages in cell cycle.


A
Original
Enlarged
Merge
eGFP-RalA
TfR
Rab11
GM130
γ-adaptin
+Nocodazole
+Tannic acid
+Brefeldin A
B
α-tubulin
Phalloidin
C
D
Density
Fractions
1 3 5 7 9 10 11 12 13 14 15 17 19 21
RalA
Rab4
EEA1
Syntaxin-6
Sec8
Nek2
Akt
E
Interphase
Mitosis
Anaphase
Telophase/
Cytokinesis

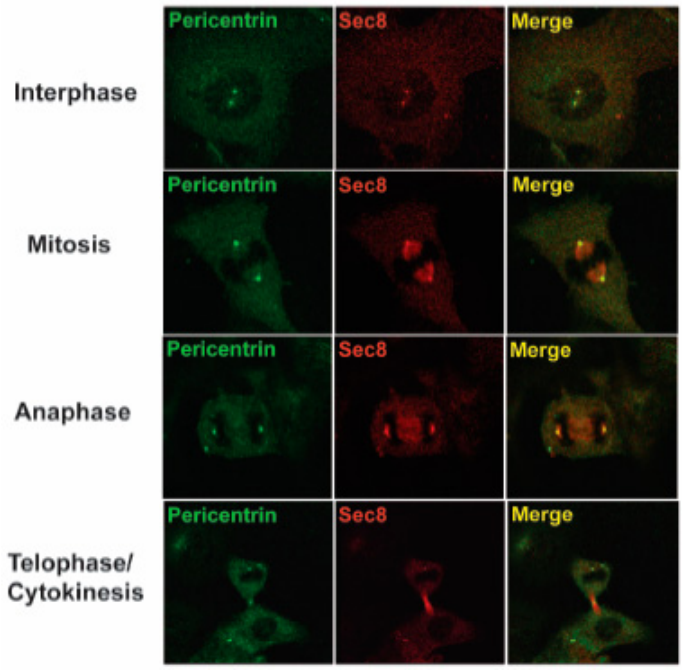
endosome recycling, suggesting the similar properties of the vesicular membranes marked by these GTPases. Taken together, we conclude that RalA localizes to a subset of recycling endosomes in both polarized and non-polarized cells, suggesting an important role of RalA in regulating vesicle trafficking via the endocytotic recycling route.

Interestingly, we observed that RalA underwent cell cycle-dependent re-localization (Figure 2.2E) similar to certain RE proteins involved in vesicle trafficking during cytokinesis [17, 18]. Co-staining of cells with α-tubulin revealed that RalA localized predominantly to the plasma membrane in mitosis, during which endosome recycling stops. However, upon initiation of anaphase, RalA was targeted to the ingestion furrow and later the intracellular bridge. Moreover, during the final step of cytokinesis, RalA localized to the abscission site, indicating a role for RalA in vesicle trafficking during the completion of cytokinesis.

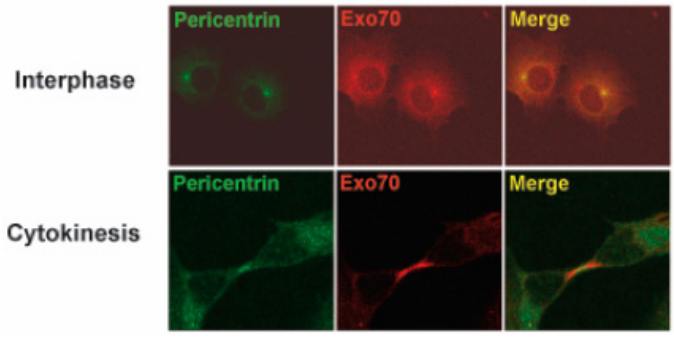
The exocyst is spatially and temporally localized during the cell cycle. RalA has been implicated in polarized trafficking through its interaction with the exocyst complex, which plays an essential role in vesicle targeting during cytokinesis in lower organisms [7-9]. We thus postulated that the exocyst complex may also facilitate polarized vesicle trafficking during mammalian cytokinesis. Immuno-fluorescence microscopy showed a punctate localization throughout the cell for the exocyst subunit Sec8, which accumulated in the juxtanuclear region where it co-localized with the centrosome marker pericentrin. During mitosis and cytokinesis, Sec8 associated with the mitotic apparatus including the

Figure 2.3. The exocyst is spatially and temporally regulated during the cell cycle. **(A)** Cell cycle dependent re-localization of Sec8. Cos-1 cells were stained using antibodies against Pericentrin (green) and Sec8 (red). Sec8 localizes to the centrosome during interphase, and then concentrates on mitotic spindles (mitosis), central spindles (anaphase), and the midbody (cytokinesis). **(B)** Subcellular localization of Exo70. Cos-1 cells were stained with Pericentrin (green) antibody and Exo70 (red) antiserum. **(C)** Cos-1 cells were transfected with HA-Sec5 full length and stained with antibody against Pericentrin (green) and HA (red). Cytokinetic cells with different expression level of Sec5 were shown. **(D)** Biochemical evidence that the exocyst associates with the midbody. Midbody isolation was performed according to standard methods [19] with cytokinetic CHO cells or unsynchronized cell as control. Total cell lysate and pellets of the above experiment were subjected to SDS-PAGE, followed by Western blot for indicated proteins.

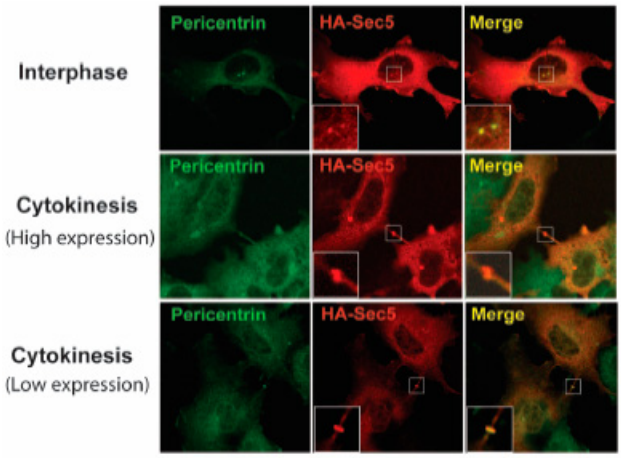
A


Interphase
Mitosis
Anaphase
Telophase/
Cytokinesis
Pericentrin
Sec8
Merge

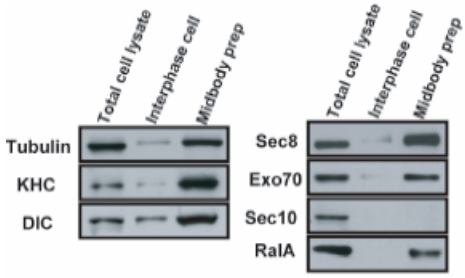
B


Interphase
Cytokinesis
Pericentrin
Exo70
Merge

C


Interphase
Cytokinesis
(High expression)
Cytokinesis
(Low expression)
Pericentrin
HA-Sec5
Merge

D


Total cell lysate
Interphase cell
Midbody prep
Tubulin
KHC
DIC
Sec8
Exo70
Sec10
RalA

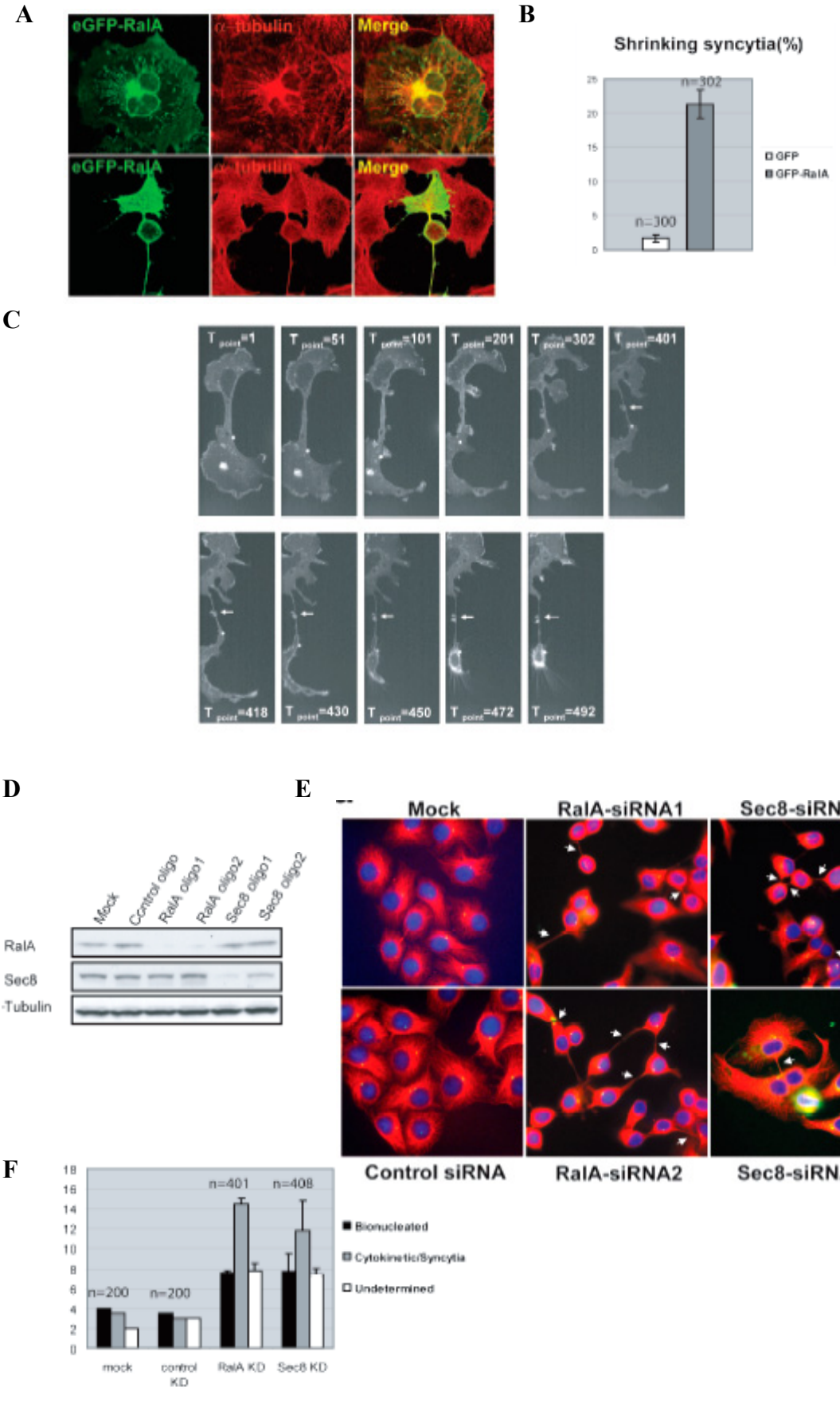
spindle poles, and in cytokinetic structures including the central spindles and the midbody (Figure 2.3A). The same results were obtained using a different Sec8 antibody (data not shown). Another exocyst protein, Exo70, is also localized around and at the centrosome and later the midbody (Figure 2.3B), as is the over-expressed exocyst protein Sec5 (Figure 2.3C) and Sec8 (data not shown). Notably, ectopic expression revealed that the exocyst proteins are enriched in the abscission site, indicating that the exocyst may also regulate vesicular trafficking to the abscission site of the dividing cells. This enrichment was not visualized by endogenous protein staining, possibly due to epitope masking, a common technical difficulty in midbody staining. A recent proteomics study profiling midbody-associated proteins revealed that the exocyst subunit Sec3 is associated with the midbody [19]. Indeed, we found that the exocyst proteins and RalA are present in biochemically isolated midbody from synchronized CHO cells (Figure 2.3D), further suggesting that these proteins may participate in membrane trafficking during cytokinesis.

Disruption of RalA-exocyst function causes late stage cytokinesis failure. The dynamic re-localization of RalA and the exocyst to cytokinetic structures led us to investigate their potential roles in cell cycle progress. We observed that over-expression of RalA in Cos-1 cells leads to a moderate increase of bi-nucleated cells (Figure 2.4A, upper panel), a phenotype commonly resulting from cytokinesis failure. However, we also observed that a significant fraction of the cells form two-cell syncytia connected by microtubule bundles, and one cell body shrinks and often detaches from the culture substrate (Figure 2.4A, lower panel and Figure 2.4B). To investigate this phenomenon in

detail, we performed live cell imaging on Cos-1 cells over-expressing eGFP-RalA (Figure 2.4C). We found that upon initiation of cytokinesis, RalA was transported to the intracellular bridge in vesicular bodies and also localized to the plasma membrane of the cleavage furrow, similar to what was visualized in fixed cells. As cytokinesis proceeds, RalA associates with the abscission sites localized at the center of the intracellular bridge. However, the cells remain interconnected for a long period of time (≥6 hours after initiation of cytokinesis), as determined by the persistent abscission site marked by eGFP-RalA, and finally one cell collapses, possibly due to the increased cellular tension or triggering of apoptosis after failed cytokinesis [20].

To further address the function of RalA and the exocyst in cytokinesis, we used siRNA-mediated knockdown to deplete cellular expression of RalA and Sec8 in HeLa cells. The efficiency of the knockdown was verified by fluorescent oligos (data not shown) and Western blot (Figure 2.4D). When examined by immuno-fluorescence microscopy, both Sec8 and RalA knockdown in HeLa cells led to an increased number of bi-nucleated cells. However, a significant percentage of cells formed syncytia with two, three, or four cells that are connected with prolonged yet stretched intracellular bridges (Figure 2.4E, F). These multi-cell syncytia may arise from failed cytokinesis following a second round of mitosis of the individual cells, since one of the connected cells can re-enter mitosis. Taken together, the data indicate that disruption of the RalA-exocyst function leads to a late stage cytokinesis failure, likely resulted from incomplete abscission.

Figure 2.4 Disruption of RalA-exocyst function results in late stage cytokinesis failure. (A) Over-expression of RalA leads to formation of binucleated cells (upper panel) and cell syncytia with persistent intracellular bridge (lower panel). Cos-1 cells were transfected with eGFP-RalA for 36 hours, and stained with α-tubulin (red) antibody. **(B)** Percentage of cells that form syncytia when RalA was over-expressed. **(C)** Live cell images were taken on Cos-1 cells expressing eGFP-RalA at 30 seconds interval. Arrow indicates the abscission site at the middle of the intracellular bridge. **(D)** HeLa cells were transfected with 100 nM of the indicated siRNA oligos and lysed in SDS contained buffer. Cellular protein levels were determined by Western blot following SDS-PAGE. **(E)** HeLa cells transfected with indicated siRNA oligos were stained with antibodies against Pericentrin (green), α-tubulin (red). The nucleus was visualized by DAPI (blue); images were taken on an epi-fluorescence microscope. The arrows indicate the persistent intracellular bridges connecting daughter cells together. **(F)** Percentage of the cells after siRNA transfection showing cell cycle defects. Cells were categorized into binucleated, cytokinetic or syncytia, or undetermined if the cells are too close to be determined.


A
eGFP-RalA
α-tubulin
Merge
eGFP-RalA
α-tubulin
Merge
B
Shrinking syncytia(%)
n=302
n=300
GFP
GFP-RalA
C
T point=1
T point=51
T point=101
T point=201
T point=302
T point=401
T point=418
T point=430
T point=450
T point=472
T point=492
D
Mock
Control oligo
RalA oligo1
RalA oligo2
Sec8 oligo1
Sec8 oligo2
RalA
Sec8
Tubulin
E
Mock
RalA-siRNA1
Sec8-siRNA1
Control siRNA
RalA-siRNA2
Sec8-siRNA2
F
n=200
n=200
n=401
n=408
mock
control KD
RalA KD
Sec8 KD
Binucleated
Cytokinetic/Syncytia
Undetermined

Discussion

Membrane trafficking is an essential but poorly understood step in cytokinesis [3]. We report here a critical role of the exocyst, as well as its interacting GTPase RalA, in directing membrane trafficking to the key cytokinetic structures in mammalian systems. Our data suggest that besides fulfilling a general need for membrane addition, the exocyst may mediate the delivery of a specific set of vesicles to the abscission site.

The data described here support the idea that RE-derived membrane trafficking is crucial for the completion of cytokinesis [3, 18], and points to a novel mechanism underlying this polarized vesicle delivery event. We found that RalA is localized and activated on RE and RE-derived vesicles. Moreover, RalA is spatially and temporally regulated during cell division, coupled with the re-localization of exocyst proteins to cytokinetic structures. Disruptions of RalA or exocyst function lead to similar cell cycle defects. Taken together, these results suggest a role of the exocyst in targeting RE-derived vesicles via its interaction with RalA. This is somewhat different from the originally proposed function of the exocyst in regulating post-Golgi secretion/exocytosis, and may reflect cross-talk between the secretory pathway and endocytic recycling pathway during cytokinesis. On the other hand, several recent papers report the association of exocyst proteins with the RE-localized adaptor AP-1B in polarized cells [21], and the interaction between Rab11 and Sec15 [22], as well as the presence of Sec10 on RE membrane [23]. Hence, we reason that this regulatory function in RE trafficking may also represent the adaptation of the exocyst to a more specialized vesicle trafficking route.

These data lead to the hypothesis that the exocyst targets RalA-localized vesicles to key cytokinetic structures. Indeed, we found that the exocyst protein Sec8 dictates the function of Sec5, which in turn bridges RalA to the compartments marked by Sec8 (unpublished observation). However, the events upstream of this polarized vesicle delivery are not completely understood, as both Sec8 and Sec5 seem to be mobilized in a RalA-independent manner. The classic Rappaport experiment [24] and additional recent studies indicated that the centrosome is able to influence polarized vesicle trafficking from the RE [18, 25, 26]. We find that both RalA and the exocyst are associated with the centrosome, whereas disruption of their function causes defects in the late stage of cytokinesis, similar to defects resulting from a loss of centrosome function. This relationship between the centrosome and the exocyst was also suggested by a recent study (26). Interestingly, many centrosomal proteins have been reported to associate with central spindles and/or the midbody [19, 27, 28]. These data further suggest that the exocyst is the molecular link that directs RalA-containing vesicles to the centrosome and centrosome-related structures. This idea coincides with a recent study by Gromley et al., who reported that the exocyst complex is recruited to the midbody by the MT motor protein MKLP and the centrosomal protein centriolin [29]. It is also noteworthy that other RE-related proteins, such as FIP3 [18] and Dynamin II [28], show localization and function similar to the exocyst. Therefore, it will be interesting to learn whether they function in a parallel or linear pathway in endocytotic recycling.

The mechanisms controlling mammalian cell abscission are poorly studied. Nevertheless, several groups have suggested that the centrosome plays a crucial role in the completion of cytokinesis, likely via its transient re-localization to the future abscission site [30-32]. This repositioning may direct a specific set of vesicles to seal off the abscission site or facilitate recruiting key signaling proteins [33]. It is tempting to hypothesize that the exocyst may provide the essential specificity of this process. One possibility is that RalA, via the exocyst, may regulate the delivery of a group of proteins essential for abscission. Interestingly, two recent reports [18] demonstrated the role of Rab11 and Arf6 in cytokinesis, along with the involvement of the exocyst. Hence, the abundance of RE-localized GTPases that are involved in cytokinesis suggests that they regulate different pools of vesicles derived from RE. On the other hand, it is also possible that RalA itself may have a signaling role in cell separation. In correlation with our finding that active RalA localizes to the centrosome and the abscission site, Wu et. al. reported that Aurora-A, a centrosome-localized kinase essential for cell cycle regulation, is able to activate RalA [34]. In addition, RalBP1, a Ral effector containing a Rho GAP domain, was first identified as a centrosome antigen [35]. These observations may recapitulate the signaling pathways regulating cell separation in yeast [36], raising an intriguing hypothesis that the repositioning of the centrosome may bring spatially segregated signaling molecules together at a specific time during the cell cycle.

In summary, we have demonstrated an important role for the mammalian exocyst in completion of cytokinesis, by targeting RE-derived vesicle trafficking through its interaction with the GTPase RalA. In addition, our results suggest a potential mechanism

by which the centrosome may control the terminal stage of cytokinesis. Since RE-mediated trafficking is also implicated in other polarized cellular events and regulated exocytosis in specialized cells [37], it will be interesting to investigate how the exocyst may facilitate these trafficking events in response to the specific signal cues.

Materials and Methods

Constructs- Full length Sec5 was obtained from a 3T3-L1 cDNA library by PCR, and completely sequenced, then cloned into the pKH3 vector [38]. Sec5 RBD (1-120) and RalBP1 RBD (397-519) were cloned into peYFP-C1 vector (BD biosciences). Sec5 RBD T11A was generated using site directed mutagenesis (Stratagene). RalA variants were cloned into pK-Flag vector or peGFP-C3 vector (BD biosciences).

Cell culture, transfection, and inhibitors- Cos-1 and Hela cells were grown in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U ml^{-1} streptomycin (GIBCO). CHO cells were grown in DMEM/F-12 medium supplemented with the 10% fetal bovine serum and 100 U ml^{-1} streptomycin. Cos-1 cells were transfected using Fugene 6 (Roche) according to the manufacturer's directions. All chemicals and inhibitors were from Sigma. Cells were treated at 37^{o}C with 33μM nocodazole for 2 hours, 2μM cytochalasin D for 1 hour, 2μM Brefeldin A for 2 hours, or 0.5% tannic acids for 10 minutes.

Immunofluorescence and antibodies- Cells were grown on glass coverslips and washed with PBS before fixation. After fixation with methanol at -20^{o}C for 3 minutes, cells were re-hydrated in PBS, and then blocked with 1% BSA and 1% chicken albumin. For RBD experiments, cells were fixed with 10% formalin at room temperature for 10 minutes, neutralized with 100 mM Glycine/PBS, and then permeablized with 0.5% Tx-100 before blocking. RalA localization remains the same in different fixation conditions. Primary antibodies used were α-tubulin (1:500, mAb), Flag (1:500, rabbit IgG), and γ-adaptin(1:200, mAb) from Sigma; Pericentrin (1:100, rabbit IgG) from Abcam; TfR (1:500, mAb), Rab11 (1:50, rabbit IgG) from alpha Diagnostic; GM130 (1:100, mAb) from BD biosciences; Sec8 (1:100, mAb) from Stressgen; HA (1:500, mAb or rabbit IgG) from Santa Cruz biotechnology. Monoclonal anti-Exo70 was described previously (18). Alexa-Fluor conjugated goat anti-mouse/rabbit secondary antibodies, Alexa-Fluor conjugated Phalloidin, and Vector Shield mounting medium were from Molecular Probe. The following antibodies were used in biochemical assays: RalA, Rab4, Nek2, EEA1 mAbs were from BD biosciences; rabbit anti-Exo84 was from Orbigen; rabbit anti-Sec10 was kindly provided by Dr.Wei Guo (University of Pennsylvania).

Western blots- Cells were washed with PBS and lysed for 30 minutes at 4^{o}C in buffer (buffer A) containing 100mM Tris (pH 7.5), 1% NP-40, 135mM NaCl, 1mM EDTA, 1.0mM sodium orthovanadate, 10mM NaF, and protease inhibitor tablets (Roche). The lysates were subjected to SDS-PAGE and transferred to nitrocellulose. Individual proteins were detected with specific antibodies and visualized by blotting with HRP-conjugated secondary antibodies.

Midbody prep- CHO cell midbody prep was performed according to a previous study [19]. The pellet fraction of interphase cells and cytokinetic cells treated with taxol and jasplakinolide were subjected to SDS-PAGE and blotted with specific antibodies.

Opti-prep gradient- Cos cells were washed with PBS, homogenized in HES buffer (20mM Hepes pH 7.4, 1mM EDTA, 250 mM Sucrose) 10 times with a ball bearing homogenizer (Wheaton), and spun at 3,000g for 3 minutes to generate post-nuclear supernatant (PNS). To generate a 10-20-30% continuous gradient, 1.2 ml PNS was mixed 1:1 with 60% iodixanol (Opti-Prep), and layered under 1.3 ml 20% iodixanol and 1.2 ml 10% iodixanol, respectively. The gradient was spun at 72,000 rpm in a fixed angle NVT90 rotor for 3 hours at 4^{o}C and fractionated into 25 fractions. Equal volume of each fraction was loaded in SDS-PAGE.

siRNA knockdown- The following siRNA oligos (Invitrogen) were used. RalA: 5'-CCAAGGGUCAGAAUUCUUU-3' (oligo-1 sense), 5'-GCUAAUGUUGACAAGGUAU-3' (oligo-2 sense); Sec8: 5'-CCUUGAUACCUCUCACUA U -3' (oligo-1 sense), 5'- GCUUUCUCCAAUCUUUCU A -3' (oligo-2 sense); Control oligos with medium GC content or fluorescent labeling were also from Invitrogen. 100 nM oligos were transfected into Hela cells using oligofectamine according to the manufactory's instruction. After 3 days, cells were trypsinized and re-plated at low density, and a second round of knockdown was

performed. Cells were either harvested in SDS-PAGE sample buffer for Western blot or fixed for immuno-fluorescent microscopy.

Time-lapse microscopy- Cos cells expressing eGFP-RalA were treated with 100 nM nocodazole for 16 hours. The mitotic cells were harvested by centrifuge and released into cell cycle for 30-40 minutes before being imaged at 37^0C using an upright fluorescent spinning disk microscope (Leica). Images were taken under a 63X oil lens at 30 second intervals.

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Chapter 3

The Activation of RalA is Required for Insulin Stimulated Glut4 Trafficking to the Plasma Membrane via the Exocyst and the Motor Protein Myo1c

Introduction

Insulin stimulates glucose transport in fat and muscle cells through a process of regulated vesicle recycling, in which the insulin-responsive glucose transporter Glut4 is translocated from intracellular sites to the plasma membrane [1, 2]. In the basal state, Glut4 undergoes endocytosis via endocytic compartments and is subsequently sorted into specialized storage vesicles that rapidly translocate to the plasma membrane upon insulin stimulation. These vesicles then dock and fuse at specific sites on the plasma membrane, resulting in extracellular exposure of the transporter. Despite much attention, the precise mechanisms that link insulin signaling to these trafficking events remain poorly understood [3].

We recently demonstrated that the exocyst complex plays a pivotal role in insulin-stimulated glucose transport by facilitating the docking of Glut4 vesicles to the plasma membrane [4, 5]. The exocyst is an evolutionarily conserved vesicle tethering complex comprised of eight subunits [6, 7]. The assembled complex mediates the initial recognition between exocytic vesicles and the target membrane, under the control of integrated signal inputs from different

small GTPases that are spatially regulated [8]. In yeast, Rho family GTPases anchor part of the exocyst on the plasma membrane through Exo70p and Sec3p; whereas the vesicle-localized GTPase Sec4p recruits other components to the exocytic vesicles via its effector Sec15p [6, 9, 10]. Sec4p also forms a complex with the unconventional myosin motor Myo2p and its light chain Mlc1p, which then deliver the vesicles along a cytoskeletal track [10-14].

Despite its conserved presence through evolution, whether the exocyst may adopt similar schemes to regulate vesicle targeting in eukaryotes remains uncertain [15, 16]. A mammalian functional homolog of Sec4 that regulates exocyst-mediated vesicle targeting has not yet been identified; and even less is known about the involvement of the cytoskeleton and molecular motors. In adipocytes, activation of TC10 on the plasma membrane in response to insulin mobilizes at least part of the exocyst [4, 17], a process essential for the docking and fusion of Glut4 vesicles [4, 5]. However, the mechanisms by which Glut4 vesicles are directed to and recognize the exocyst remain unknown. Central to this question is whether a second, vesicle-localized G protein may integrate signaling from the insulin receptor, and subsequently coordinate transport machineries, including the exocyst and motor proteins, for Glut4 delivery.

The small GTPase RalA has emerged as a convergent point of cellular signaling and vesicular trafficking [18]. Ral GTPases (RalA and RalB) interact with the exocyst subunits Sec5 and Exo84 [19-21]; RalA has higher affinity for these proteins than does RalB [22]. Recent studies have reported the regulatory role of RalA, but not RalB, as well

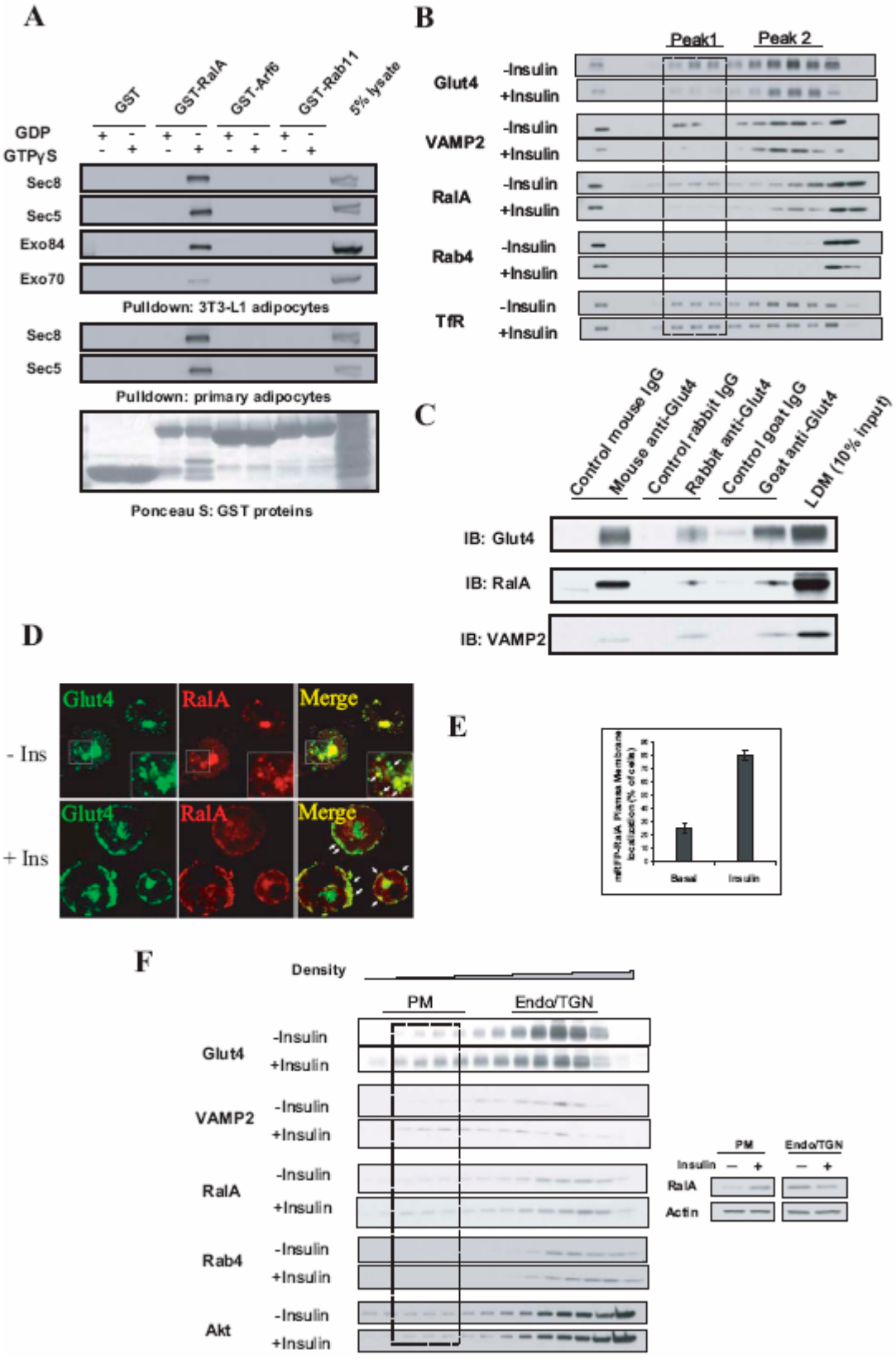
as the involvement of the exocyst, in varied processes, including E-cadherin exocytosis [22], oncogenic transformation [23], and cytokinesis[24-26]. Here we report that insulin-stimulated glucose transport requires the activation of RalA and its interactions with both the exocyst complex and the unconventional myosin Myo1c complex.

Results

RalA resides in Glut4 vesicles. While the exocyst complex is required for the trafficking of specialized Glut4 storage vesicles in response to insulin, how the vesicle recognizes exocyst components is unknown. To identify proteins that might be involved in vesicle:exocyst recognition, we screened for vesicular-localized GTPases that might fulfill some of these roles [16] in adipocytes by pull down, followed by western blotting. RalA, but not Arf6 or Rab11, specifically precipitated the exocyst proteins including Sec5 and 8, Exo84 and Exo70 in a GTP-dependent manner in both 3T3-L1 adipocytes and primary mouse adipocytes (Figure 3.1A).

RalA has been reported to localize to vesicles derived from the recycling endosome [22, 24], an organelle implicated in the sorting of insulin-responsive Glut4 vesicles [27]. To test whether RalA may associate with Glut4 vesicles, low density microsome (LDM) fractions were prepared from 3T3-L1 adipocytes treated without or with insulin, and then further fractionated on opti-prep density gradients to separate different pools of intracellular vesicles. Glut4 was separated into two peaks; the first (peak 1) was only found in untreated cells, indicating that this population of Glut4 vesicles translocated to

Figure 3.1. RalA is a Glut4 vesicle associated GTPase connecting the native exocyst complex. (A) RalA, but not Arf6 or Rab11 interacts with the adipocyte exocyst. Cell lysates prepared from 3T3-L1 adipocytes (upper) or primary adipocytes (lower) were incubated with Glutathione beads coupled with the indicated GST-fusion proteins that were pre-loaded with GDP or GTPγS. Bound proteins were subjected to SDS-PAGE and Western Blotting (WB). **(B)** RalA co-fractionates with an insulin-responsive Glut4 population. Low density microsome (LDM) fractions prepared from 3T3-L1 adipocytes treated without or with insulin for 10 minutes were fractionated using a self-generated Opti-prep gradient. Fractions were subjected to SDS-PAGE starting from light density fractions and WB. The first lane represented LDM fractions. **(C)** RalA is present in affinity purified Glut4 membranes. LDM fractions prepared from 3T3-L1 adipocytes were incubated with the indicated antibodies pre-coupled to Protein A/G beads. After washing extensively, bound proteins were eluted with detergent, subjected to SDS-PAGE, and WB. **(D)** RalA co-localizes with Glut4. 3T3-L1 adipocytes were transfected with plasmids encoding mRFP-RalA and Glut4-eGFP, and stimulated without or with insulin for 30 minutes. Cells were fixed and subjected to confocal microscopy; compiled images of individual cells from different fields were presented for illustration purposes. Arrows indicated co-localization. **(E)** Quantification of cells in (D) with plasma membrane (PM) localization of RalA. n= 4 x 100. **(F)** Endogenous RalA translocates to the PM in response to insulin. Left: 3T3-L1 adipocytes were homogenized after stimulation without or with insulin for 20 minutes. Cell homogenates were fractionated using a self generated Opti-prep (10-20-30%). Right: fractions of plasma membrane (in the box) and Endo/TGN (the peak fraction and the neighboring two) were pooled and subjected to SDS-PAGE on the same gel, followed by WB analysis.


A
GST
GST-RalA
GST-Arf6
GST-Rab11
5% lysate
GDP
GTPγS
Sec8
Sec5
Exo84
Exo70
Pulldown: 3T3-L1 adipocytes
Sec8
Sec5
Pulldown: primary adipocytes
Ponceau S: GST proteins
B
Peak1
Peak 2
Glut4
VAMP2
RalA
Rab4
TfR
-Insulin
+Insulin
C
Control mouse IgG
Mouse anti-Glut4
Control rabbit IgG
Rabbit anti-Glut4
Control goat IgG
Goat anti-Glut4
LDM (10% input)
IB: Glut4
IB: RalA
IB: VAMP2
D
Glut4
RalA
Merge
- Ins
+ Ins
E
Basal
Insulin
F
Density
PM
Endo/TGN
Glut4
VAMP2
RalA
Rab4
Akt
-Insulin
+Insulin
Insulin
RalA
Actin

the plasma membrane in response to insulin. The second peak was less insulin sensitive. RalA co-fractionated with the first Glut4-containing pool of vesicles, as did the v-SNARE VAMP2, which regulates plasma membrane fusion of Glut4 vesicles (Figure 3.1B). Both RalA and VAMP2 disappeared in response to insulin, indicating that these proteins may physically associate with Glut4 vesicles. To further explore this possibility, we immuno-purified Glut4 vesicles from LDM fractions of 3T3-L1 adipocytes with three different antibodies. RalA was present in each preparation of these immunopurified vesicles, but not in those found with control antibodies (Figure 3.1C). We also examined the localization of Glut4 and RalA by microscopy. 3T3-L1 adipocytes were transfected with an mRFP-tagged RalA construct and an eGFP-tagged Glut4 construct. mRFP-RalA co-localized with Glut4-eGFP in perinuclear regions in un-stimulated 3T3-L1 adipocytes. Insulin promoted the translocation of both proteins to the plasma membrane (Figure 3.1D, E). Consistent with these observations, fractionation of 3T3-L1 adipocyte homogenates revealed that endogenous RalA relocated to plasma membrane fractions upon insulin stimulation, as did Glut4 and VAMP2 (Figure 3.1F). Taken together, these data suggested that RalA associates with insulin-responsive Glut4 vesicles.

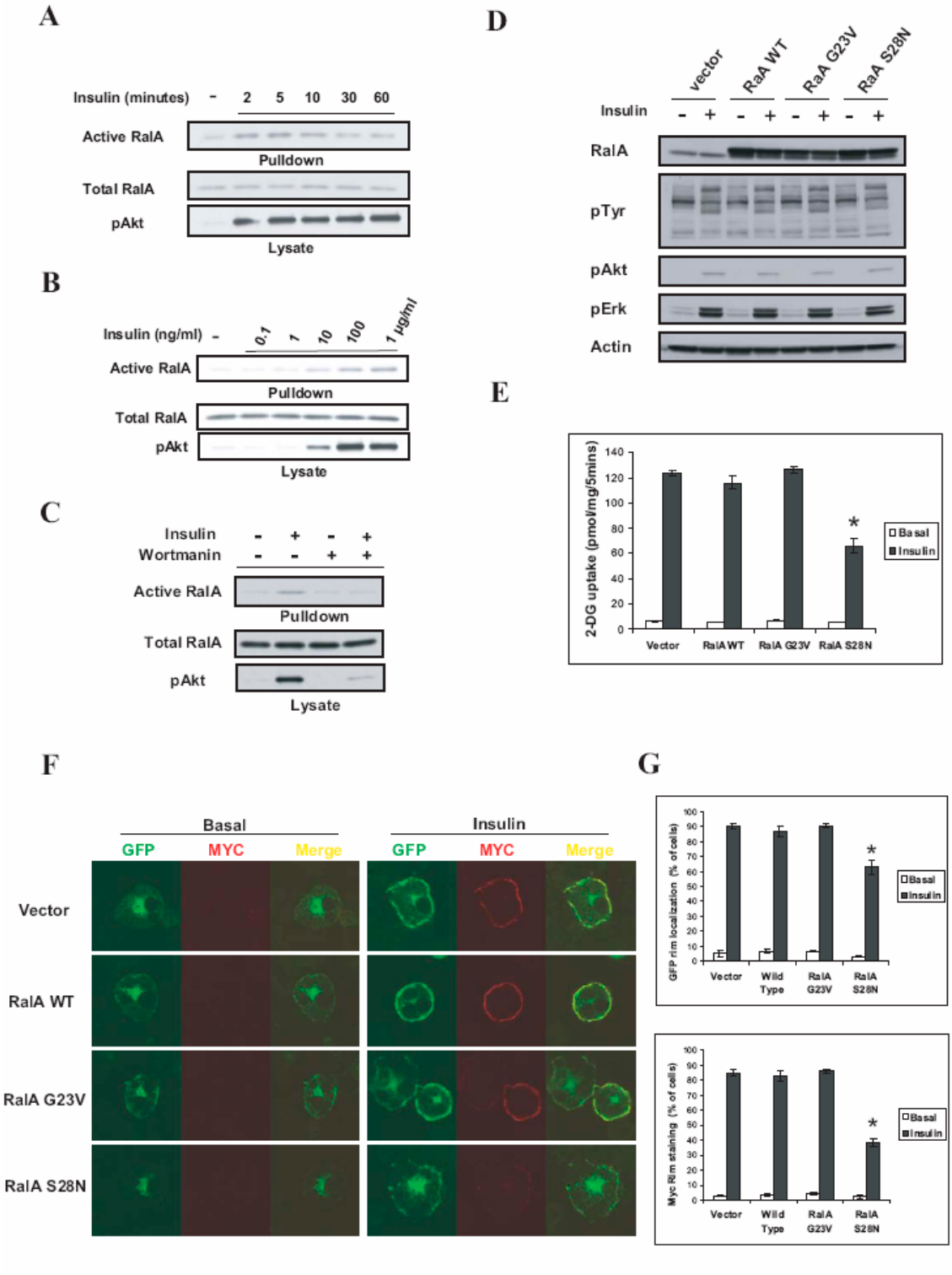
RalA is activated by insulin, a step required for targeting Glut4 to the plasma membrane. The association of RalA with Glut4 vesicles led us to investigate whether insulin may regulate the activity of the G protein, and whether its activity state plays a role in Glut4 trafficking. 3T3-L1 adipocytes were treated with insulin for up to 60 minutes and at different doses, and RalA activity was assessed by pull down with an immobilized Ral binding domain (RBD) of RalBP1. Insulin stimulated the activation of

RalA in a time and dose-dependent manner, with maximal activation occurring at about 5 minutes (Figure 3.2A, B). Intriguingly, insulin activation of RalA was blocked by pre-treatment of cells with wortmannin, an inhibitor of PI 3-Kinase that is critical for insulin-stimulated Glut4 translocation (Figure 3.2C).

To determine whether activation of RalA is necessary for insulin-stimulated glucose transport, we introduced wild type, constitutively active ($G^{23}V$), and dominant negative ($S^{28}N$) forms of RalA into 3T3-L1 adipocytes, and assayed 2-deoxy-glucose uptake after stimulation with insulin. The expression of all three proteins was verified by Western Blotting, and was without effect on insulin-stimulated tyrosine phosphorylation, Erk phosphorylation, or Akt phosphorylation (Figure 3.2D). While wild-type and constitutively active RalA mutants had no effect on insulin-stimulated glucose uptake, dominant negative RalA produced a ~ 40% reduction in the effect of the hormone (Figure 3.2E). A similar inhibitory effect was observed after expression of RalA ($G^{26}A$), another dominant negative mutant form of RalA (data not shown). Considering the 50-60% transfection efficiency achieved in these experiments, these results indicated that the activity of RalA is required for the stimulation of glucose uptake by insulin.

To further examine the effects of these mutants on Glut4 trafficking, we co-expressed the RalA constructs with a Myc-Glut4-eGFP reporter, in which a triple Myc tag was introduced into the exo-facial loop of Glut4 to monitor its extracellular exposure after vesicle docking and fusion. Consistent with the glucose uptake assay, dominant negative RalA inhibited insulin-stimulated Glut4 translocation as well as its subsequent fusion

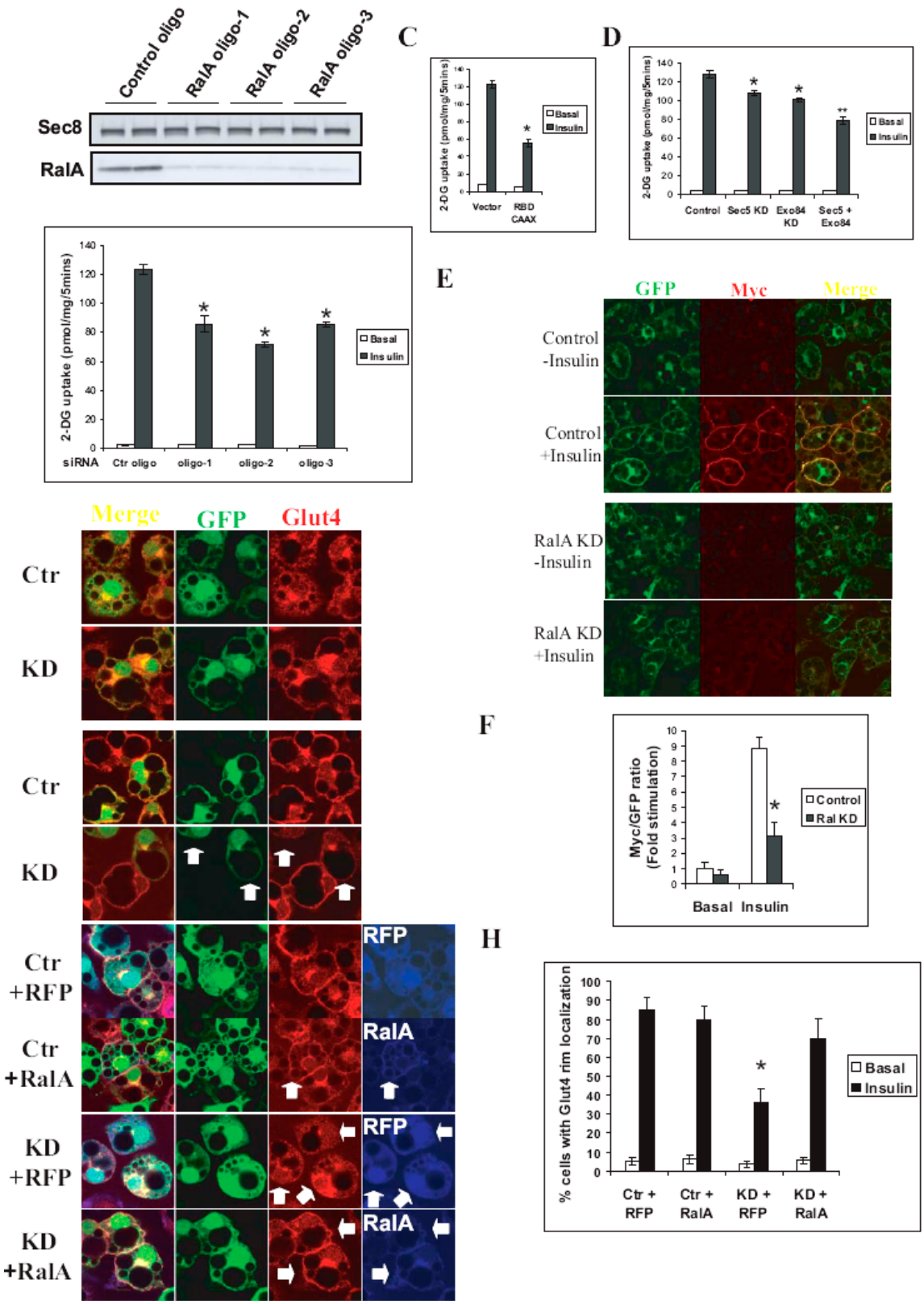
Figure 3.2. Activation of RalA by insulin is involved in targeting Glut4 to the plasma membrane. (A) Time course of RalA activation by insulin. 3T3-L1 adipocytes were stimulated as indicated and lysed. Cell lysates were subjected to pull down using GST-RalBP1 RBD. RalA from the pulldown, or total RalA and pAkt in the lysates were determined by WB following SDS-PAGE separately. The experiment shown was representative of five independent experiments. About 1-3% of total cellular RalA was present in the pulldown. **(B)** Dose-dependent activation of RalA by insulin. 3T3-L1 adipocytes were treated with indicated doses of insulin for 5 minutes. An assay similar to that described in (A) was performed to determine the level of active RalA. The experiment shown was representative of three independent experiments. **(C)** RalA activation by insulin is wortmannin-sensitive. 3T3-L1 adipocytes were pre-treated with 100 nM wortmannin for 30 minutes, and then stimulated with insulin as indicated. An assay identical to that described in (A) was performed. The experiment shown was representative of five independent experiments. **(D)** Expression of RalA of different forms has no effects on proximal insulin signaling. 3T3-L1 adipocytes were electroporated with plasmids encoding proteins indicated. Cell lysate were subjected to SDS-PAGE and WB. **(E)** Dominant negative RalA inhibits insulin-stimulated 2-deoxy-glucose (2-DG) uptake. 3T3-L1 adipocytes expressing the indicated proteins were subjected to a 2-DG uptake assay. Asterisk: $p < 0.001$. The experiment shown was representative of four independent experiments. **(F)** Dominant negative RalA inhibits Glut4 trafficking. 3T3-L1 adipocytes were co-transfected with plasmids encoding myc-Glut4-eGFP and indicated RalA mutants. Cells were stimulated without or with insulin for 30 minutes, fixed without permeablization, stained with Myc antibody, and examined by confocal microscopy. (**G, H)** Quantification of cells in (E) that had plasma membrane rim staining of GFP or Myc. Asterisk: $p<0.001$. $n = 3 \times 100$ for both basal and stimulated states.


A
Insulin (minutes) - 2 5 10 30 60
Active RalA
Pulldown
Total RalA
pAkt
Lysate
B
Insulin (ng/ml) - 0.1 1 10 100 1 µg/ml
Active RalA
Pulldown
Total RalA
pAkt
Lysate
C
Insulin - + - +
Wortmanin - - + +
Active RalA
Pulldown
Total RalA
pAkt
Lysate
D
vector
RaA WT
RaA G23V
RaA S28N
Insulin - + - + - + - +
RalA
pTyr
pAkt
pErk
Actin
E
2-DG uptake (pmol/mg/5mins)
140
120
100
80
60
40
20
0
Vector
RalA WT
RalA G23V
RalA S28N
Basal
Insulin
F
Basal
Insulin
GFP
MYC
Merge
Vector
RalA WT
RalA G23V
RalA S28N
G
GFP rim localization (% of cells)
Vector
Wild Type
RalA G23V
RalA S28N
Basal
Insulin
Myc Rim staining (% of cells)
Vector
Wild Type
RalA G23V
RalA S28N
Basal
Insulin

with the plasma membrane (Figure 3.2F), as quantified by the plasma membrane rim localization of both GFP and Myc in non-permeablized cells (Figure 3.2G). In contrast, the wild-type and constitutively active mutants were without effect.

To further explore the role of RalA in insulin action, siRNA-mediated knockdown was carried out to deplete cellular RalA. Three different siRNA oligos against RalA were used to achieve approximately 90% knockdown of the protein (Figure 3.3A, data not shown). Depletion of RalA had no apparent effect on insulin-stimulated tyrosine phosphorylation or Akt phosphorylation (data not shown). However, addition of the siRNA oligos to 3T3-L1 adipocytes attenuated insulin-stimulated 2-deoxy-glucose uptake in a trend that correlated with the level of RalA reduction (Figure 3.3B), with the second oligo sequence being the most effective. Considering that Glut4 only accounts for a portion of glucose uptake in 3T3-L1 adipocytes due to the presence of Glut1 transporter and knockdown cannot eliminate cellular function of the target protein, the inhibition of glucose uptake from RalA knockdown is likely to represent a major blockage of insulin action. We then employed an inhibitory probe, comprised of Sec5 RBD and the C-terminal targeting motif of RalA, to block the downstream of activated RalA. Expression of this probe also led to a significant inhibition of glucose uptake (Figure 3.3C). Moreover, knockdown of Sec5 and Exo84, the two exocyst subunits that interact with RalA, also inhibited glucose uptake in response to insulin (Figure 3.3D), consistent with what was previously reported for other exocyst proteins in regulating Glut4 exocytosis [5], further suggesting that the unification of the exocyst is required for glucose transport.

Figure 3.3. Loss of RalA attenuates insulin stimulated glucose transport. (A,B) Loss of RalA inhibits insulin-stimulated glucose uptake. 3T3-L1 adipocytes transfected with the indicated siRNA oligos were subjected to WB (A) using indicated antibodies and 2-DG uptake assay (B). Asterisk: $p<0.003$, double asterisks: $p<0.0005$. The experiment shown was representative of three independent experiments. **(C)** Inhibition the downstream of activated RalA blocks insulin-stimulated glucose uptake. Adipocytes transfected with vector or Sec5-RBD fused with C-terminus of RalA were subjected to 2-DG uptake assay as described above. Asterisk: $p<0.0005$. The experiment shown was reprehensive of three independent experiments. **(D)** Loss of Sec5 or Exo84, the two exocyst subunits interacting with RalA, inhibits insulin stimulated 2-deoxy-glucose (2-DG) uptake. 3T3-L1 adipocytes transfected with the indicated siRNA oligos were subjected to 2-DG uptake assay. Radioactive 2-DG incorporation was determined after normalizing to protein concentration. Asterisk: $p<0.02$, double asterisks: $p<0.001$. **(E)** Loss of RalA inhibits trafficking of Myc-Glut4-eGFP. 3T3-L1 adipocytes stably expressing Myc-Glut4-eGFP were transfected with control or RalA siRNA oligos. After insulin treatment, cells were fixed without permeablization, stained with Myc antibody, and examined by confocal microscopy. **(F)** Quantification of (D). GFP or Myc fluorescent signals of cells in (D) were quantified with fluorescent spectrometer. Error bars represented standard deviation mean from eight replicated samples for each condition tested. The experiment shown was representative of three independent experiments. **(G)** Restoration of Glut4 trafficking from RalA knockdown by re-introducing RNAi-resistant RalA. 3T3-L1 adipocytes were infected first with lentiviruses expressing control or RalA knockdown shRNA for 6 days, and then with lentiviruses expressing RFP or mRFP-RalA. ShRNA expressing were monitored by expression of GFP. After starvation, cells were stimulated as indicated, and processed for immuno-fluorescence to stain endogenous Glut4 (red). Expression of cDNA was monitored by RFP signal (pseudo-colored in blue) **(H)** Quantification of cells with Glut4 rim staining in (G). Error bars represented standard deviation from 4 different experiments. Asterisk: $p<0.001$, $n = 4 \times 100$ for both basal and insulin-stimulated states.


Control oligo
RalA oligo-1
RalA oligo-2
RalA oligo-3
Sec8
RalA
C
2-DG uptake (pmol/mg/5mins)
Basal
Insulin
Vector
RBD CAAX
D
Control
Sec5 KD
Exo84 KD
Sec5 + Exo84
siRNA
Ctr oligo
oligo-1
oligo-2
oligo-3
E
GFP
Myc
Merge
Control -Insulin
Control +Insulin
RalA KD -Insulin
RalA KD +Insulin
Merge
GFP
Glut4
Ctr
KD
F
Myc/GFP ratio (Fold stimulation)
Control
Ral KD
Basal
Insulin
RFP
RalA
Ctr +RFP
Ctr +RalA
KD +RFP
KD +RalA
H
% cells with Glut4 rim localization
Basal
Insulin
Ctr + RFP
Ctr + RalA
KD + RFP
KD + RalA

To quantitatively study the role of RalA in insulin-stimulated Glut4 trafficking, we employed an adipocyte line in which Myc-Glut4-eGFP was stably expressed. RalA knockdown inhibited insulin-stimulated plasma membrane insertion of Glut4 (Figure 3.3E), as determined by total Myc staining without cell permeablization versus eGFP fluorescence (Figure 3.3F). Taken together with the dominant interfering mutant experiments presented above (Figure 3.2E-G), these data suggested a required role foRalA in Glut4 trafficking, likely in both the translocation and docking of Glut4 vesicles.

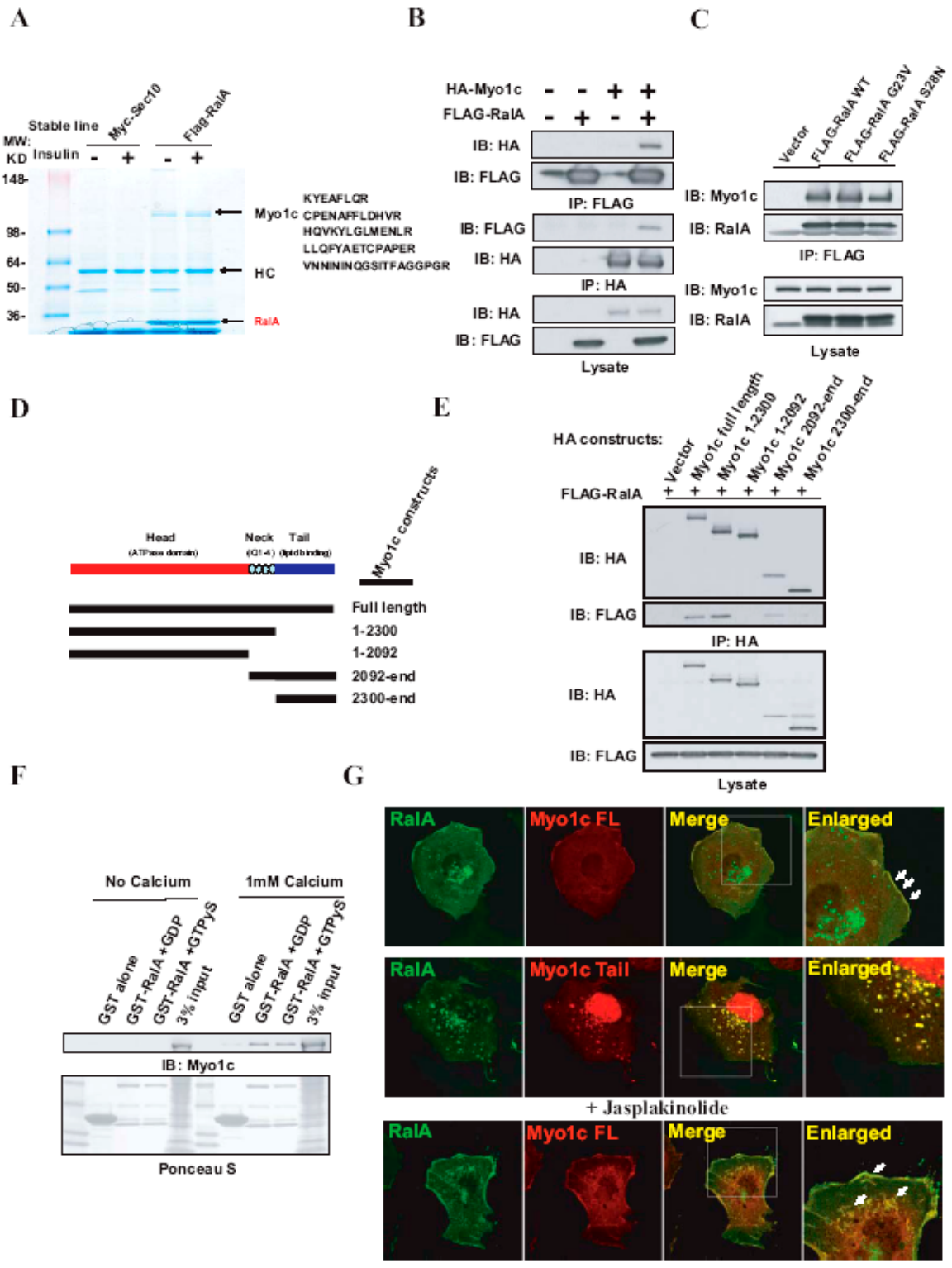
We finally examined whether the defects in Glut4 trafficking from RalA knockdown can be reversed by re-introducing RalA. Due to the different time course of RNA interference and cDNA expression, we employed a lentiviral system to achieve stable knockdown over time. Viral infection was then monitored by the expression of eGFP that was encoded in the same viral construct. Insulin-stimulated translocation of endogenous Glut4 to the plasma membrane was similarly inhibited in adipocytes expressing shRNA against RalA, but not in control cells (Figure 3.3G, *upper panel*). The defects in RalA knockdown cells could be reverted by re-introducing the RNAi-resistent cDNA of RalA (Figure 3.3H) tagged with mRFP, but not with mRFP alone, while both proteins had little effects on Glut4 in control cells (Figure 3.3G, *lower panel*). Taken together, the data further strengthened the required role of RalA in insulin action to regulate Glut4 trafficking.

The Myo1c motor associates with RalA. The involvement of RalA in Glut4 translocation and docking is reminiscent of the yeast protein Sec4p, which coordinates

the function of the exocyst and myosin motor in exocytosis [6, 10-14]. Several lines of evidence support the involvement of motor proteins in transporting Glut4 vesicles along the cytoskeletal track [28-30], raising the possibility that they may cooperate with the exocyst during Glut4 trafficking. To identify additional proteins that may associate with RalA, we employed Mass Spectrometry following a co-immunoprecipitation experiment using a FLAG-RalA stable cell line. One protein was identified by both MS and MS/MS analysis as Myo1c, an unconventional myosin that is thought to associate with and transport Glut4 vesicles [29, 30] (Figure 3.4A). This interaction was confirmed by reciprocal co-immunoprecipitation with recombinant proteins (Figure 3.4B). The interaction between Myo1c and RalA in adipocytes was independent of the nucleotide binding status of RalA (Figure 3.4C).

To delineate the domains in Myo1c that mediate its interaction with RalA, we generated a series of Myo1c truncation mutants (Figure 3.4D), and performed co-immunoprecipitation experiments. These studies revealed that the IQ domains of Myo1c are required for interacting with RalA (Figure 3.4E). Both the lipid-binding tail domain and IQ domains of Myosin I proteins have been implicated in mediating recruitment of the unconventional Myosins to membrane compartments, while the latter cases appear to involve calcium-sensitive Myosin light chains [31-34]. Consistent with this possibility, we found that the interaction between GST-RalA and Myo1c in adipocytes is sensitive to calcium (Figure 3.4F), suggesting the involvement of the light chain of Myo1c. These data raised the possibility that RalA may serve as a cargo receptor for Myo1c, linking the molecular motor to the Glut4 vesicle.

Figure 3.4. The Myo1c motor interacts with RalA. **(A)** Identification of RalA binding protein as Myo1c by Mass Spectrometry (MS). Control or FLAG-RalA stable cell lines were lysed and subjected to immuno-precipitation (IP). Bound proteins were separated using SDS-PAGE; protein bands were sliced and subjected to both MS and MS/MS analysis. **(B)** Confirmation of the Mass Spectrometry results with recombinant proteins. Cos cells were transfected with plasmids encoding indicated proteins, and subjected to IP with indiated antibodies. Bound proteins were subjected to SDS-PAGE and WB. **(C)** Nucleotide-independent interaction between RalA and Myo1c in adipocytes. 3T3-L1 adipocytes transfected with the indicated RalA constructs were lysed and subject to IP using FLAG antibody. Bound proteins were subjected to SDS-PAGE and WB. **(D)** Schematics of Myo1c truncation mutants. **(E)** Mapping Myo1c domains that mediate RalA interaction. Cos cells expressing the indicated constructs were lysed, and subjected to IP using HA antibody. Bound proteins were subjected to SDS-PAGE and WB. **(F)** Calcium-sensitive interaction between GST-RalA and Myo1c. 3T3-L1 adipocytes were lysed with buffers containing 1mM $CaCl_2$ or EDTA, and incubated with the indicated GST-fusion proteins coupled to GSH beads. Bound proteins were subjected to SDS-PAGE and WB. **(G)** Sub-cellular localization of Myo1c and RalA. Cos-1 cells were transfected with eGFP-RalA wild type and RFP- Myo1c full length (FL) or Myo1c (IQT), fixed, and subjected to fluorescent microscopy. In lower panel, cells were treated with 500 nM Jasplakinolide for 1 hour.


A
Stable line
Myc-Sec10
Flag-RalA
MW:
KD
Insulin
148-
98-
64-
50-
36-
Myo1c
HC
RalA
KYEAFLQR
CPENAFFLDHVR
HQVKYLGLMENLR
LLQFYAETCPAPER
VNNINQGSITFAGGPGR
B
HA-Myo1c
FLAG-RalA
IB: HA
IB: FLAG
IP: FLAG
IB: FLAG
IB: HA
IP: HA
IB: HA
IB: FLAG
Lysate
C
Vector
FLAG-RalA WT
FLAG-RalA G23V
FLAG-RalA S28N
IB: Myo1c
IB: RalA
IP: FLAG
IB: Myo1c
IB: RalA
Lysate
D
Head
(ATPase domain)
Neck
(IQ1-6)
Tail
(lipid binding)
Myo1c constructs
Full length
1-2300
1-2092
2092-end
2300-end
E
HA constructs:
Vector
Myo1c full length
Myo1c 1-2300
Myo1c 1-2092
Myo1c 2092-end
Myo1c 2300-end
FLAG-RalA
IB: HA
IB: FLAG
IP: HA
IB: HA
IB: FLAG
Lysate
F
No Calcium
1mM Calcium
GST alone
GST-RalA +GDP
GST-RalA +GTPγS
3% input
GST alone
GST-RalA +GDP
GST-RalA +GTPγS
3% input
IB: Myo1c
Ponceau S
G
RalA
Myo1c FL
Merge
Enlarged
RalA
Myo1c Tail
Merge
Enlarged
+ Jasplakinolide
RalA
Myo1c FL
Merge
Enlarged

When examined by microscopy, RFP-tagged full length Myo1c largely co-localized with eGFP-RalA on membrane ruffles on the plasma membrane (Figure 3.4G, *upper panel*). However, deletion of the Myo1c motor domain resulted in co-localization with RalA on vesicular membranes, and reduced the plasma membrane localization of RalA (Figure 3.4G, *middle panel*). These observations suggested a transient association between Myo1c motor and RalA-vesicles when active transport is present during endocytic recycling. Indeed, treatment of cells with Jasplakinolide induced formation of excess rod-like actin filament of different length; both RalA and Myo1c localized on these filaments throughout the cell (Figure 3.4G, *lower panel*).

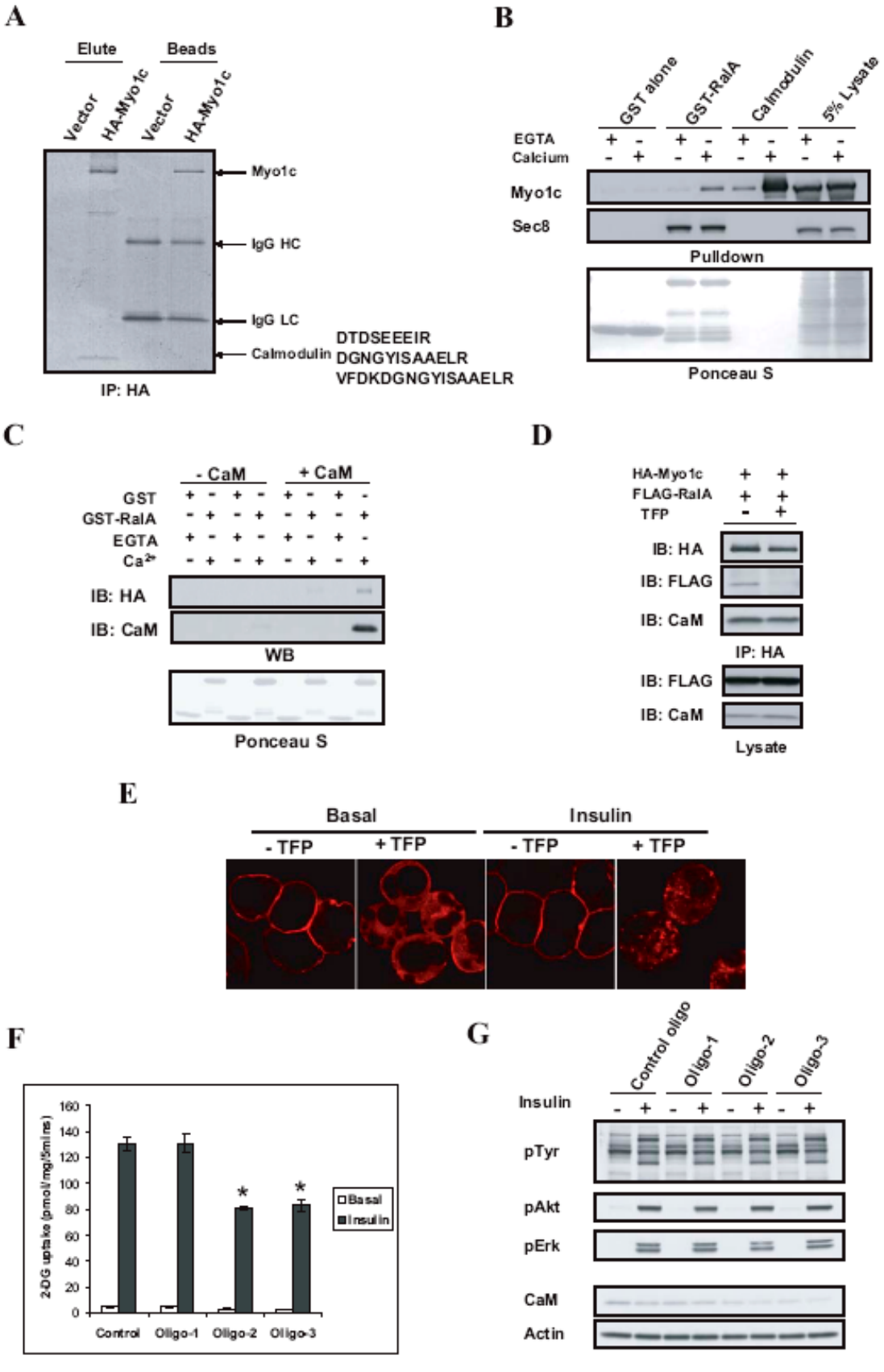
Calmodulin regulates Myo1c function in glucose transport. Studies in yeast have suggested that the transport of Sec4-localized vesicles by Myo2 is controlled by a Myo2 light chain, Mlc1 [35], which directly interacts with the first two proteins to form a ternary complex [14]. Although it remains unclear whether this ternary complex is solely responsible for Myo2 recruitment, disruption of its formation prevented Myo2 from transporting exocyst-targeted vesicles. This led us to hypothesize that Myo1c, like many molecular motors including kinesins and dyneins particularly, operates in complex with the light chains to recognize cargo receptors in regulating Glut4 trafficking. To evaluate this possibility, we expressed HA-tagged Myo1c in adipocytes, and searched for associated proteins via Mass Spectrometry as described above. We identified Calmodulin as an interacting light chain for Myo1c in adipocytes (Figure 3.5A). Co-immunoprecipitation experiments with Myo1c mutants confirmed that this interaction is also mediated via Myo1c IQ domains (data not shown). Interestingly, Calmodulin, like

Myo1c, also interacted with RalA in a nucleotide-independent manner (data not shown), as previously reported [36, 37].

As a myosin light chain, Calmodulin adopts different molecular conformations in its calcium-bound or free form, thus regulating the overall structure of the Calmodulin-Myosin complex [32]. It has been suggested that in the case of the unconventional myosin Myosin-V, calcium-bound Calmodulin leads to an "active" extended conformation of the motor complex, permitting cargo loading and transport initiation [38]. Consistent with this, we found that calcium-bound Calmodulin formed a stronger interaction with Myo1c in a pull down assay using recombinant Calmodulin, compared with the calcium-free form (Figure 3.5B). We thus performed *in vitro* assessment to test whether Calmodulin may facilitate the association between RalA and Myo1c. Indeed, GST-RalA only interacted with *in vitro* translated HA-Myo1c in the presence of calcium bound recombinant Calmodulin (Figure 3.5C). Taken together with the calcium-sensitive nature of Myo1c-RalA interaction (Figure 3.3F), these data indicated that calcium-bound Calmodulin may enhance the surface accessibility of the Myo1c motor complex, thus facilitating RalA interactions and the recognition of cargo vesicles.

To test this possibility in more detail, we evaluated the effects of the Calmodulin inhibitor trifluoperazine (TFP), which interacts with calcium-bound Calmodulin and prevents it from interacting with other proteins. Treatment of cells with TFP blocked the interaction between Myo1c and RalA (Figure 3.5D). Notably, addition of this compound to adipocytes has also been shown to block insulin stimulated plasma membrane

Figure 3.5. Calmodulin regulates Myo1c function in glucose transport. (A) Identification of Calmodulin as a Myo1c Light Chain in adipocytes. 3T3-L1 adipocytes were transfected with the indicated constructs and subjected to IP using HA antibody after lysis. Proteins in immunoprecipitates were separated by SDS-PAGE and subjected to MS and MS/MS analysis. **(B)** Calcium-dependent interaction between Calmodulin and Myo1c. 3T3-L1 adipocytes were lysed with buffers containing 1mM $CaCl_2$ or EGTA. Cell lysates were then incubated with the indicated proteins coupled to beads. Bound proteins were analyzed by WB following SDS-PAGE. **(C)** Calcium-bound Calmodulin is required for RalA-Myo1c association *in vitro*. GST or GST-RalA coupled on beads was incubated with *in vitro* translated HA-Myo1c in the absence or presence of purified Calmodulin, in either EGTA or Calcium containing buffer. Bound proteins were subjected to SDS-PAGE and WB. **(D)** The Calmodulin inhibitor TFP inhibits RalA-Myo1c interaction. Cos cells expressing the indicated constructs were treated without or with TFP for 20 minutes and subjected to anti-HA IP after lysis. Bound proteins were then analyzed by WB following SDS-PAGE. **(E)** TFP mis-localizes Myo1c from plasma membrane in adipocytes. 3T3-L1 adipocytes were treated with or without 50 μM TFP for 20 minutes, and then stimulated with or without insulin for 20minutes before fixation. Cells were stained with Myo1c antibody and subjected to microscopy. **(F)** Loss of Calmodulin inhibits insulin-stimulated glucose uptake. 3T3-L1 adipocytes transfected with the indicated siRNA oligos were subjected to 2-DG uptake assay. Note that oligo-1 failed to deplete Calmodulin, as shown in Figure 5f. Asterisk: $p<0.003$. The experiment shown was representative of three independent experiments. **(G)** Knockdown of Calmodulin is without effect on proximal insulin signaling. 3T3-L1 adipocytes transfected with the indicated siRNA oligos were treated without or with insulin for 30 minutes and lysed. Cell lysates were subjected to SDS-PAGE and WB.


A
Elute
Beads
Vector
HA-Myo1c
Vector
HA-Myo1c
Myo1c
IgG HC
IgG LC
Calmodulin
IP: HA
DTDSEEEIR
DGNGYISAAELR
VFDKDGNGYISAAELR
B
GST alone
GST-RalA
Calmodulin
5% Lysate
EGTA
Calcium
Myo1c
Sec8
Pulldown
Ponceau S
C
- CaM
+ CaM
GST
GST-RalA
EGTA
Ca2+
IB: HA
IB: CaM
WB
Ponceau S
D
HA-Myo1c
FLAG-RalA
TFP
IB: HA
IB: FLAG
IB: CaM
IP: HA
IB: FLAG
IB: CaM
Lysate
E
Basal
Insulin
- TFP
+ TFP
- TFP
+ TFP
F
2-DG uptake (pmol/mg/5mins)
160
140
120
100
80
60
40
20
0
Basal
Insulin
Control
Oligo-1
Oligo-2
Oligo-3
G
Control oligo
Oligo-1
Oligo-2
Oligo-3
Insulin
pTyr
pAkt
pErk
CaM
Actin

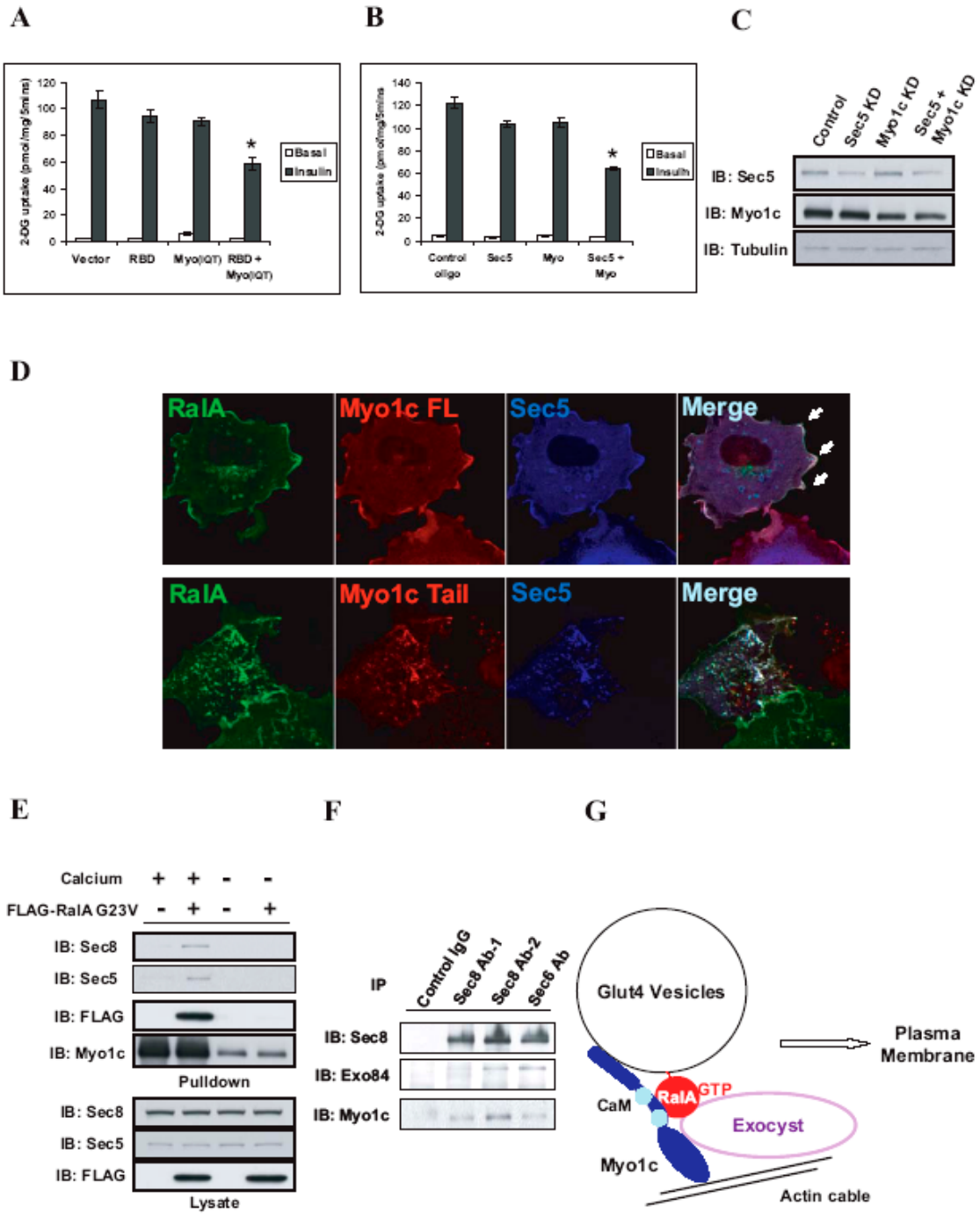
translocation of Glut4, but not Glut1, suggesting a selective role of Calmodulin in insulin action [39]. Besides inhibition of Glut4 trafficking (data not shown), we also found that TFP treatment inhibited the localization of endogenous Myo1c to the plasma membrane (Figure 3.5E), reinforcing the regulatory role of Calmodulin on Myo1c in Glut4 trafficking. Furthermore, these data were consistent with the hypothesis that Myo1c, in complex with Calmodulin, recognizes RalA as a cargo receptor on Glut4 vesicles. We next depleted Calmodulin by siRNA-mediated knockdown as an alternative means to disrupt the Myo1c-RalA complex, followed by evaluation of insulin-stimulated glucose transport. Loss of Calmodulin resulted in a 40-50% inhibition of 2-deoxy-glucose uptake stimulated by insulin (Figure 3.5F). This inhibition appeared to correlate with the Calmodulin expression level, and was not due to any detectable blockade of proximal insulin signaling events (Figure 3.5G). The data is consistent with the proposed role of Calmodulin in insulin-regulated glucose transport, although we cannot rule out the involvement of other long term effects of Calmodulin knockdown.

Coordination between the exocyst and Myo1c regulates Glut4 trafficking to the plasma membrane. Genetic studies in yeast have revealed that the combination of Myo2 and exocyst partial loss-of-function mutants resulted in synthetic lethality [40, 41], suggesting that vesicle delivery and docking are closely coupled by these proteins for efficient transport [11]. We thus hypothesized that efficient Glut4 trafficking may also require concerted actions between Myo1c and the exocyst, in the process ensuring the velocity and specificity of transport. To test this, we set out to determine whether disruption of Myosin and the exocyst together may augment inhibitory effects on glucose

transport. We first employed dominant negative mutants of Myo1c and Sec5. Overexpression of the Myo1c C-terminus, Myo (IQT) [29] and Sec5 Ral binding domain (RBD) [19] together led to a synergistic inhibition of insulin-stimulated 2-deoxy-glucose uptake in 3T3-L1 adipocytes, compared to expression of either of the mutants alone (Figure 3.6A). Similar results were obtained with siRNA-mediated knockdown (Figure 3.6B), in which cellular Sec5, Myo1c, or both were partially depleted (Figure 3.6C), implying that these proteins may coordinate with each other in regulating Glut4 vesicle targeting. Consistent with this idea, we found that the fast exchanging mutant ($F^{39}L$) of RalA induced vesicular localization of HA-Sec5. Moreover, both proteins co-localized with endogenous Myo1c on membrane ruffles on the plasma membrane (Figure 3.6D, *upper panel*). However, the Myo (IQT) fragement, which lacked the motor domain, decorated intracellular tubule-vesicular structures where both RalA and Sec5 were localized (Figure 3.6D, *lower panel*). Taken together, these data suggest that Myo1c could recognize RalA-resided vesicles bound with some exocyst subunits, further indicating a cooperative effort of these two complexes in targeting RalA-associated vesicles.

To further determine the molecular basis of this process, we tested whether some of the exocyst complex may physically associate with the Myo1c complex, thus bridging Glut4 vesicles to the assembled exocyst complex prior to vesicle fusion with the plasma membrane. We employed two approaches to capture the association of these proteins. First, we introduced into cells a constitutively active mutant of RalA ($G^{23}V$) to induce its interaction with the exocyst. We then lysed cells and incubated lysates with Calmodulin-

Figure 3.6. Coordination between the exocyst and Myo1c regulates Glut4 trafficking to the plasma membrane. (A) Synergistic inhibition of insulin-stimulated glucose uptake by Myo1c (IQT) and Sec5 RBD. 3T3-L1 adipocytes expressing the indicated constructs were subjected to 2-DG uptake assay. Asterisk: $p<0.004$. The experiment shown was representative of three independent experiments. **(B)** Synergistic inhibition of insulin-stimulated glucose uptake by Myo1c and Sec5 knockdown. 3T3-L1 adipocytes were transfected with the indicated siRNA oligos and subjected to 2-DG uptake assay. Asterisk: $p<0.001$. The experiment shown was representative of four independent experiments. **(C)** WB of knockdown. 3T3-L1 adipocytes transfected with the indicated siRNA oligos were subjected to SDS-PAGE and WB after lysis. **(D)** Co-localization of RalA, Sec5, and Myo1c. Cos-1 cells were transfected with eGFP-RalA $F^{39}L$, HA-Sec5, or Myo1c (IQT) when indicated. Cells were fixed, stained with HA or Myo1c antibody and subjected to microscopy. **(E)** Calcium-sensitive interaction of Calmodulin, RalA, and the exocyst complex. Cos cells transfected with the indicated constructs were lysed with buffers containing calcium and EGTA. Cell lysates were incubated with Calmodulin beads, and bound proteins were determined by WB after SDS-PAGE. **(F)** Myo1c interacts with the exocyst. 3T3-L1 adipocytes were cross-linked by the cell permeable crosslinker DSP, lysed in SDS containing buffer. Cell lysates were subjected to IP with the indicated antibodies, and immunoprecipitates were analyzed by WB after SDS-PAGE. **(G)** Hypothetical model of the role of RalA, the exocyst, and the Myo1c complex in insulin-stimulated Glut4 trafficking.


A
B
C
2-DG uptake (pmol/mg/5min)
Basal
Insulin
Vector
RBD
Myo(IQT)
RBD + Myo(IQT)
Control oligo
Sec5
Myo
Sec5 + Myo
Control
Sec5 KD
Myo1c KD
Sec5 + Myo1c KD
IB: Sec5
IB: Myo1c
IB: Tubulin
D
RalA
Myo1c FL
Sec5
Merge
RalA
Myo1c Tail
Sec5
Merge
E
F
G
Calcium
FLAG-RalA G23V
IB: Sec8
IB: Sec5
IB: FLAG
IB: Myo1c
Pulldown
IB: Sec8
IB: Sec5
IB: FLAG
Lysate
IP
Control IgG
Sec8 Ab-1
Sec8 Ab-2
Sec6 Ab
IB: Sec8
IB: Exo84
IB: Myo1c
Glut4 Vesicles
Plasma Membrane
CaM
RalA
GTP
Exocyst
Myo1c
Actin cable

coupled beads. Consistent with the observation that calcium-bound Calmodulin mediates the association between Myo1c and RalA; calcium-activated Calmodulin interacted with RalA, and subsequently with the exocyst proteins (Figure 3.6E). As an alternative approach to stabilize protein-protein interactions, we performed a crosslinking experiment in non-starved 3T3-L1 adipocytes using the cell permeable cross-linker DSP. Myo1c was found to associate with the exocyst complex in a co-immunoprecipitation experiment using three different antibodies (Figure 3.6F). Taken together, these data suggested a possible mode of action in which the exocyst complex and the Myo1c complex cooperate with each other in targeting Glut4 vesicles to the plasma membrane (Figure 3.6G).

Discussion

Insulin-stimulated glucose transport involves the concerted actions of different cellular machineries that regulate distinct steps in Glut4 vesicle trafficking [1-3]. Here we present evidence that the translocation of Glut4 vesicles and their subsequent docking with the plasma membrane are closely coupled, through coordination between the Myo1c motor complex and the exocyst complex, in a manner that is regulated by the small GTPase RalA.

RalA interacts with the adipocyte exocyst complex in a GTP-dependent manner, and undergoes activation in response to insulin. Moreover, RalA also interacts with a motor complex containing Myo1c, although the latter interaction is GTP-independent.

Disruption of the function of RalA or of any of its interacting proteins in these complexes attenuates insulin-stimulated glucose transport. The role of RalA is reminiscent of the function of the yeast GTPase Sec4p, which links the yeast exocyst complex with the unconventional myosin complex Myo2p/Mlc1p during vesicle transport [6, 10, 12-14]. RalA may coordinate the activity of a molecular motor and vesicle tethering complex, in the process ensuring the velocity and specificity of Glut4 vesicle transport. Taken together with the regulation of mammalian exocyst by activated TC10 on the plasma membrane, the data further supports the intriguing scheme in which the exocyst is bridged to plasma membrane and vesicles via spatially segregated activation of different G-proteins. This nevertheless demonstrates the extremely conserved nature of fundamental vesicle trafficking processes. It is also noteworthy that the yeast exocyst has been linked to the plasma membrane SNARE complex via Sec1p [42]; and that the homologous proteins, including Munc18c and Syntaxin 4, are known to mediate the fusion of Glut4 vesicles [3]. Therefore, it is intriguing to speculate that the exocyst may also couple the Syntaxin 4-Munc18c complex to facilitate the plasma membrane fusion of Glut4 vesicles.

Our study supports the requirement of the Myo1c motor in delivery of Glut4 vesicles. In previous studies this molecular motor was detected in Glut4 vesicles and proposed to deliver them to the cell membrane [29]. Knockdown of Myo1c or overexpression of dominant negative forms of the protein blocks insulin-stimulated glucose transport [29, 30]. The binding of calmodulin to Myo1c infers a regulatory role as the Myosin light chain in this process, suggesting the requirement for a functional myosin complex.

Calmodulin modulates the association between RalA and Myo1c through the IQ motifs, in a manner that is dependent on calcium binding to Calmodulin. These results are consistent with the hypothesis that Calmodulin may regulate cargo loading and transport initiation of the Myosin motor, further suggesting that Myo1c may recognize RalA as a cargo receptor on Glut4 vesicles. Importantly, these infer potential regulatory mechanisms for Myo1c cargo recognition, which might be controlled by signals that lead to oscillation of calcium concentration or modification on calcium. Both light chain modification and cargo binding have been implicated in regulating myosin motility [38], raising the question of whether these may contribute to the relocation of Myo1c in response to insulin [29].

Despite adopting similar regulatory schemes which involve spatial regulation of different G-proteins and motor complexes in vesicle targeting, the mammalian exocyst appears to have a more specialized function than its yeast counterpart. Besides regulating Glut4 trafficking, evidence suggests that the eukaryotic exocyst is involved in endocytic recycling stimulated by extracellular signals [43], but not secretion or general endocytic recycling [44, 45]. Hence, it is tempting to speculate that activation of RalA is part of the signaling pathways that permit the mobilization of exocyst in physiological settings. In this regard, delineating the precise mechanism that activates RalA will be of interest. Our observation that RalA activation by insulin is PI 3-kinase dependent may indicate the existence of a putative Ral GEF or GAP protein that is regulated by PI 3-kinase signaling; similar to the scenario that has been proposed for the Rab GAP AS160 [46]. Alternatively, RalA may be activated locally when it is being transported to the specific sites on the

plasma membrane, in a manner resembling the activation of vesicle-associated ARF6 [47].

Recent studies indicate that PI 3-kinase signaling may control the docking and fusion steps of Glut4 vesicles, while PI 3-kinase independent signals may regulate the translocation of Glut4 towards the plasma membrane and the subsequent docking step, likely also involving cytoskeletal proteins [4, 29, 48]. However, whether and how these signaling pathways may converge remains poorly understood. Our data suggest that Glut4 vesicle docking/tethering by the exocyst complex may represent one convergent point of insulin signaling pathways, via activation of the two GTPases TC10 and RalA at different cellular compartments.

Materials and Methods

Constructs- Full length Myo1c was obtained from a 3T3-L1 cDNA library by PCR, completely sequenced, and then cloned into a pKH3 vector. Myc-Glut4-eGFP constructs was a generous gift from Dr. Jonathan Bogan (Yale). RFP cDNA was kindly provided by Dr. Roger Tsien (UCSD). Other constructs have been described previously[24].

Cell culture, transfection, and inhibitors- Cos-1 and CHO-IR cells were maintained as described previously [49]. Culture of 3T3-L1 pre-adipocytes and differentiation were performed as described [5]. Cos-1 cells were transfected using Fugene 6 (Roche).

Electroporation of adipocytes, isolation of mouse primary adipocytes, and viral infection of CHO-IR have been described previously[49]. Dithiobis(succinimidyl)propionate (DSP) was obtained from Pierce. Calmodulin conjugated beads were from Stratagene. Other chemicals and inhibitors were purchased from Sigma.

Immunofluorescence and antibodies- Cells were plated on glass coverslips and washed with PBS before fixation with 10% formalin at room temperature for 10 minutes. After neutralizing with 100 mM Glycine/PBS, cells were permeablized with 0.5% Tx-100, and then blocked with 1% BSA and 1% chicken albumin. When indicated, cells were stained without detergent permeabilization. Rabbit anti-Myo1c was kindly provided by Dr. Peter Gillespie (Oregon Health & Science University). Other primary antibodies used were: Glut4 (1:100, rabbit IgG) from alpha Diagnostic; α-6 Integrin (1:200, rat mAb) from BD biosciences; Myc (1:500, mAb or rabbit IgG) from Santa Cruz biotechnology. Alexa-Fluor conjugated goat anti-mouse/rabbit secondary antibodies, Alexa-Fluor conjugated Phalloidin, and Vector Shield mounting medium were from Molecular Probe. The following antibodies were used in biochemical assays: VAMP2 (1:500, mAb) from Synaptic System; pTyr 4G10 (1:2000, mAb) and Calmodulin (1:500, mAb) from Upstate; pAkt (1:500, pAb) and pErk (1:2000, mAb) from Cell Signaling. Goat anti-Glut4 was purchased from Santa Cruz Biotechnology; mouse anti-Glut4 was purchased from Biogenesis. Other antibodies have been described before [24].

Fractionation and Glut4 membrane purification- Homogenization of 3T3-L1 adipocytes and generation of post-nuclear supernatant (PNS) or low density microsome (LDM)

fractions were performed as described previously [5]. To further resolve the LDM fraction, LDM pellets were resuspended, mixed with Opti-prep to generate a start solution (12% iodixanol), and spun in a NVT-90 rotor for 75 minutes. Whole cell fractionation with 10-20-30% opti-prep has been described previously [24]. To purify Glut4 enriched membranes, adipocyte homogenates were spun at 50,000g to clear the PM and HDM membranes, and incubated with different antibodies that were pre-conjugated to protein A/G beads. After extensive washing, purified membrane proteins were eluted with 1% SDS and 1% Tx-100 without reducing agents.

Biochemical assays and western blotting- Cell lysis, co-immunoprecipitation and western blotting were performed essentially as described previously[49]. When indicated, EDTA was omitted from lysis buffer and 1mM $CaCl_2$ was supplemented. Additional information could be found in supplementary information. Nucleotide loading of GST-GTPases was achieved by incubating proteins with 50 mM GDP or GTPγS after stripping with EDTA. Loading was stopped by addition of 10 mM $MgCl_2$. For RalA activation assay, cell were lysed in pulldown buffer (100mM Tris (pH7.5), 1% NP-40, 0.1% Deoxycolate, 130 mM NaCl, 5mM $MgCl_2$, 1mM Na_3VO_4, 10 mM NaF, supplemented with complete protease inhibitor (Roche)). Cell lysates were cleared by spinning at top speed for 10 minutes on a bench-top centrifuge, and then passing through a 0.22 μM filter. After normalizing protein concentrations by Bradford assay, cell lysates were mixed with GST-RalBP1 agarose beads (Upstate) for 30-45 minutes. After washing, proteins on beads and cell lysates were subjected to WB analysis to determine the level of active RalA. *in vitro* translation of HA-Myo1c was performed using TNT Sp6 protein expression system

(Promega) according to the manufacturer's instruction. *in vitro* binding was performed similarly as previously reported. Briefly, GST-RalA coupled on beads was first mixed without or with 5μg of purified Calmodulin (Sigma) in buffer containing 50mM Tris, pH 7.5; 200 mM KCl; 1mM $MgCl_2$; 0.1% Tx-100; and 10% Glycerol) in the presence of 1 mM $CaCl_2$ or EGTA, 50μl of *in vitro* translocation product was then added. After 1h incubation at 4 ^{0}C, beads were washed three times, and bound proteins were subjected to SDS-PAGE and western blotting. Crosslinking of adipocytes was performed similarly as previously reported [6]. Briefly, after washing with PBS, adipocytes were treated with 200μg/ml DSP for 30 minutes at room temperature. Cells were extracted with buffer containing 1% SDS after cross-linking was quenched, and cell lysates were diluted 10 times after incubating at 55 ^{0}C for 10 minutes. Co-immunoprecipitation was performed as described above.

siRNA knockdown, 2-DG uptake, and statistics- Electroporation of adipocytes with siRNA (Stealth, Invitrogen) and insulin-stimulated 2-Deoxyglucose uptake were performed as previously reported [5]. Control oligo (GCGCCAAGCUUAGACGAUGAGCUUU) was designed as to not targeting any sequences in the mouse genome. The following oligos are used to target specific proteins:

ACAAGCCCAAGGGUCAGAAUUCUUU (RalA-1 sense)

GCGCGCCAACGUUGACAAGGUAUUU (RalA-2 sense)

UAACUAUGUGGAGACGUCUGCUAAA (RalA-3 sense)

GGCAGAAUGGAUGUCUGCGAGUAAA (Sec5 sense)

CGUCAAAGACAAUCCGCCCAUGAAA (Exo84 sense)

GAGCAGAUUGCUGAAUUCAAGGAAG (CaM-1 sense)

GAAUUCAAGGAAGCUUUCUCCCUAU (CaM-2 sense)

AAGACACAGAUAGCGAAGAAGAGAU (CaM-3 sense)

ACAGUGUGACUGGGUUUCUGGAUAA (Myo1c Sense)

For lentiviral knockdown construct, shRNA sequences (Sense: 5'-GATCCCCGCCAACGTTGACAAGGTATTTCAAGAGAATACCTTGTCAACGTTGGCTTTTTGGAAA; anti-sense: 5'-AGCTTTTCCAAAAAGCCAACGTTGACAAGGTATTCTCTTGAAATACCTTGTCAACGTTGGCGGG were generated based on the stem sequence of siRNA RalA-2 (GCGCGCCAACGUUGACAAGGUAUUU. Overlapping sequences were underlined), which gave the most efficient knockdown. The annealed DNA oligos were ligated into pSilencer vector (Ambion). The knockdown cassette were isolated by polymerase chain reaction using 5'-TTTTTCTAGAGTTTTCCCAGTCACGAC and 5'-TTTTCTCGAGGAGTTAGCTCACTCATTAGGC as primers, and introduced into viral vector using XbaI and XhoI sites. Insertion was fully sequenced by automatic sequencing (University of Michigan).

Glucose incorporation was determined after normalizing with protein concentration measured by the Bradford assay. Standard t-test (2-tailed) was performed to analyze the data. For 2-DG uptake assays, error bars represented standard deviation mean from three replicated samples for each condition tested. Error bars elsewhere represented standard deviation from at least three independent experiments.

Lentiviral work- Lentiviral expression system in adipocytes has been described before [49]. FG12 lentiviral vector for shRNA delivery was kindly provided by Dr. David Baltimore. shRNA sequences were first ligated into pSilencer vector (Ambion). Adipocytes were first infected with FG12 virus for 6 days, and then infected with virus expressing either mRFP or mRFP-RalA for 3-4 days before they were processed for experiments.

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Chapter 4

Dissecting the Architecture of the Exocyst with RalA Mutants

Introduction

Insulin stimulates glucose uptake into cells in muscle and adipose tissue by promoting the rapid translocation of the glucose transporter Glut4 to the plasma membrane [1]; a process governed by several signaling cascades and facilitated by numerous transport machineries [2, 3]. Furthermore, insulin-stimulated Glut4 trafficking represents one of the best characterized models of regulated endocytic recycling [4], which can be readily monitored in specialized, post-mitotic insulin-responsive cells such as the 3T3-L1 adipocytes [2]. While the exact mechanisms governing the specificity and efficiency of this vesicle transport event remain to be fully elucidated, recent studies have implicated the vesicle tethering complex exocyst in this process [5-7].

The exocyst is an evolutionarily conserved protein complex comprised of eight subunits, including Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 [8]. The primary function of the exocyst is to bridge exocytic vesicles to their target sites on the plasma membrane, and perhaps also to facilitate the subsequent membrane fusion process [9]. Recent structural studies on yeast exocyst proteins have provided important insights into the function of this complex [8]. Many exocyst subunits consist of long helical coiled-coil

domains that adapt to an overall rod-like structure; thus suggesting that the exocyst complex may assemble into a stretched structure. This may enable the complex to engage exocytic vesicles from a relatively long range, in the process mediating their initial plasma membrane recognition with flexibility and perhaps reversibility [8]. In this regard, the exocyst has been implicated in a variety of vesicle targeting events in different organisms from yeast to mammals [10]. Moreover, the exocyst may also coordinate with other transport machineries including the myosin motors, the SNARE complex and their regulatory proteins, thus contributing to both the specificity and efficiency of certain vesicle transport processes [9, 11].

Genetic studies in eukaryotic systems suggest that the exocyst is primarily involved in trafficking through the recycling endosome, rather than secretory pathways [12-15]. Consistent with this notion, studies in cellular models have also supported the role of the mammalian exocyst in polarized endocytic recycling to the leading edge of migrating cells [16], the sites of phagocytosis in macrophages [17], as well as the abscission sites in cytokinetic cells [18, 19]. While these suggested a widely conserved function of the exocyst, further dissection of the mechanisms in these systems has been challenging, partly due to the secondary effects that arose from cell cycle arrest upon disruption of exocyst in dividing cells [18, 19]. This has limited our understanding in the architecture of the mammalian exocyst complex, as well as its assembly in specific trafficking processes.

Recent studies have indicated a key role of small GTPases in the assembly and mobilization of the exocyst [11]. In budding yeast, the exocyst is targeted to specific sites on the plasma membrane by Rho family GTPases; whereas Sec4 triggers the assembly of the exocyst complex on secretory vesicles [20]. The mammalian exocyst interacts with TC10 at the plasma membrane [5]; and Arf6 [21], Rab11 [22], or RalA [23] on vesicles. Among these GTPases, RalA has received much attention, as activated RalA directly interacts with two exocyst subunits, Sec5 and Exo84 [24], and thus has been proposed to regulate the assembly of the exocyst [25]. Nevertheless, how the interactions of both subunits contribute to the assembly or the function of the exocyst during vesicle targeting remains uncertain. Moreover, recent structural studies suggest that, unlike what was originally proposed, Sec5 and Exo84 appear to be competitive binding partners for RalA [26, 27]. Hence, further delineation of the action of RalA will yield much insight into the mechanism by which the exocyst facilitates certain transport events.

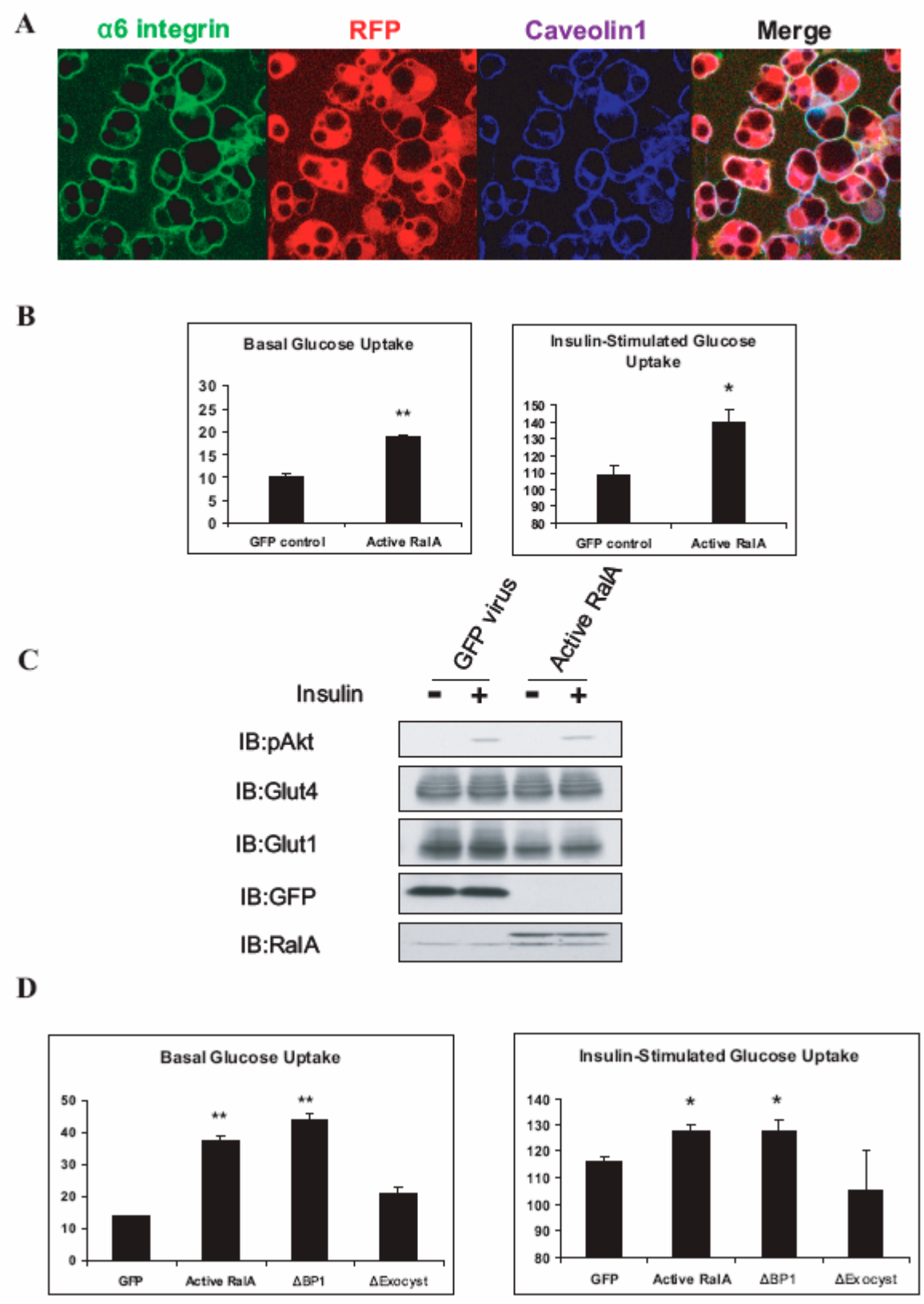
We recently reported that RalA serves a required role for insulin-stimulated glucose transport, by coordinating the function of both the exocyst and the molecular motor Myo1c [28]. In this study, we further investigated the role of RalA in organizing the exocyst in the regulation of glucose transport. By utilizing RalA mutants specifically uncoupled from one of its downstream effectors, we found that the two RalA effectors, Sec5 and Exo84, are both required for the exocyst function and further that each belongs to a sub-complex with overlapping subunits including Sec10, Sec15, and Exo70. The Sec5 branch also consists of a stable sub-complex containing Sec5, Sec6, and Sec8; whereas the remaining subunit Sec3 may serve as a potential link to the SNARE protein

Syntaxin4. Taken together, these data revealed an unexpected organization of the mammalian exocyst complex, and point to a potential mechanism by which this vesicle tethering complex exocyst is coupled to the SNARE proteins that mediate vesicle fusion.

Results

Active RalA promotes glucose uptake via exocyst interactions. We initially observed a small enhancement in insulin-stimulated Glut4 plasma membrane localization after overexpression of active RalA (G23V) was eletroporated into 3T3-L1 adipocytes [28]. However, the transfection efficiency and heterogeneity of the expression level in these experiments prevented us from further investigating the phenomenon. To address these issues, we employed the lentiviral expression system, which reportedly yielded at least 95% infection efficiency in terminally differentiated mature adipocytes with little cytotoxicity [29]. After infection of cells with lentiviruses expressing red fluorescent protein (RFP), close to 100% cells were positive for RFP fluorescence (Figure 4.1A). The adipocytes were also positive for the staining of caveolin1 or the staining of adipocyte marker α6 integrin (Figure 4.1A), suggesting lentiviral infection in adipocytes is highly effective while having little effect in the morphology or differentiation state of the infected cells. Neither did we find any observable changes in insulin signaling or glucose transport by lentivirus infection (data not shown), as previously reported by others [29]. Introduction of active RalA (G23V) into adipocytes by lentiviral infection led to a ~2 fold increase in basal glucose uptake, and ~20-30% increase in insulin-stimulated transport, compared to control adipocytes infected with viruses expressing GFP (Figure 4.1B).

Figure 4.1 Active RalA promotes glucose uptake via its interaction with the exocyst
(A) Efficient gene transduction in 3T3-L1 adipocytes with lentiviruses. Mature adipocytes infected with lentiviruses expressing red fluorescent protein (RFP, red) were subjected to immuno-fluorescent staining with α6 integrin (green) and Caveolin1 (blue) antibodies. The majority of the cells were positive for virus infection. **(B)** Active RalA promotes glucose uptake into 3T3-L1 adipocytes. Adipocytes infected with lentiviruses expressing GFP control or active RalA (G23V) were subjected to 2-deoxy-glucose (2-DG) uptake assay. Left, glucose uptake in the basal states; right, glucose uptake after insulin stimulation. Asterisk, $p<0.05$; double asterisk, $p<0.001$. Experiment shown is representative of five independent experiments. **(C)** Western blotting of cell lysates from the experiments in (B). The upper bands in RalA blot were FLAG-RalA G23V. **(D)** Active RalA promotes glucose uptake in adipocytes via exocyst interactions. 3T3-L1 adipocytes were infected with lentiviruses expressing the indicated proteins and subjected to 2-DG uptake assay. Left, glucose uptake in the basal states; right, glucose uptake after insulin stimulation. ΔBP1, RalA G23VD49N that does not interact with RalBP1; Δexocyst, RalA G23VD49E that does not interact with the exocyst. Asterisk, $p<0.05$; double asterisk, $p<0.001$. Experiment shown is representative of three independent experiments.


A
α6 integrin
RFP
Caveolin1
Merge
B
Basal Glucose Uptake
30
25
20
15
10
5
0
**
GFP control
Active RalA
Insulin-Stimulated Glucose Uptake
150
140
130
120
110
100
90
80
*
GFP control
Active RalA
C
GFP virus
Active RalA
Insulin
- + - +
IB:pAkt
IB:Glut4
IB:Glut1
IB:GFP
IB:RalA
D
Basal Glucose Uptake
50
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30
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10
0
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GFP
Active RalA
ΔBP1
ΔExocyst
Insulin-Stimulated Glucose Uptake
140
130
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GFP
Active RalA
ΔBP1
ΔExocyst

Furthermore, this increase in glucose transport was not due to enhanced insulin signaling, as no detectable changes in Tyrosine phosphorylation (data not shown) or Akt phosphorylation were observed (Figure 4.1C). In addition, the expression level of the insulin-responsive glucose transporter Glut4 was not changed, although we observed a small decrease of the Glut1 transporter (Figure 4.1C), which mediates basal glucose uptake in 3T3-L1 adipocytes and displays minor insulin responsiveness. Taken together, these data suggest that active RalA promotes glucose transport via Glut4.

This effect of active RalA resembles the gain-of-function phenotype in glucose uptake upon overexpression of several exocyst proteins in adipocytes [5, 7], leading us to test whether the enhancement in glucose transport by active RalA is dependent on the exocyst. To this end, we introduced point mutations [23] into active RalA to uncouple the G protein from its effector RalBP1 (D49N, also referred as ΔBP1), or the exocyst complex (D49E, also referred as ΔExocyst). Uncoupling active RalA from RalBP1 failed to abolish the gain-of-function effect in glucose uptake in either the basal or insulin-stimulated states. However, when uncoupled from the exocyst, active RalA failed to promote glucose uptake in either case (Figure 4.1D), suggesting that the enhancement of glucose transport by RalA is dependent on its interaction with the exocyst.

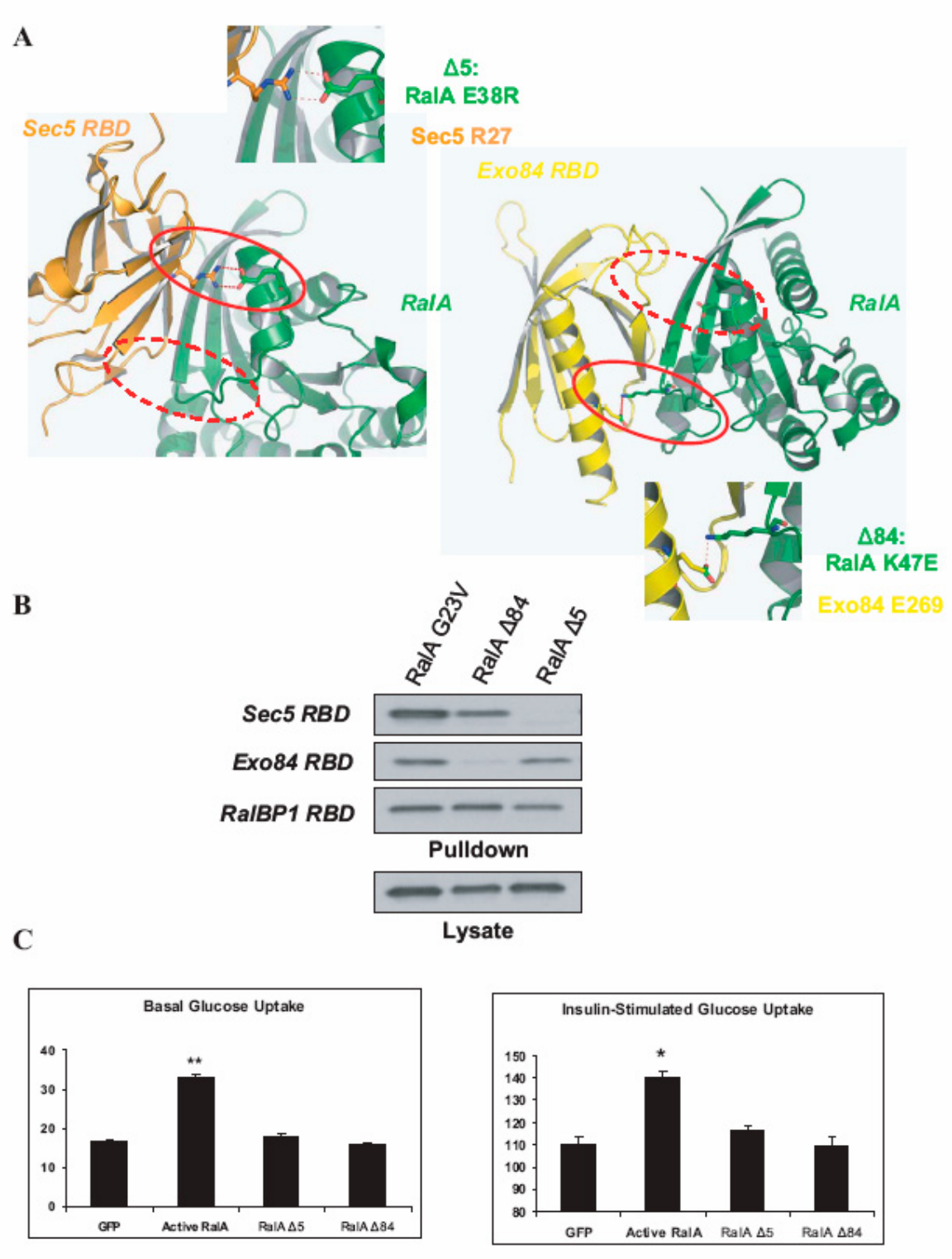
Both Sec5 and Exo84 are required for exocyst function. RalA directly interacts with two subunits of the exocyst, Sec5 and Exo84 [23, 24, 30]. However, how these interactions contribute to the organization and the function of the exocyst remains unclear [25]. Recent crystallographic studies have suggested that Sec5 and Exo84 occupy

different surface areas on the RalA protein but with overlapping interfaces (Figure 4.2A) [26, 27]. The different interaction modes would allow the generation of point mutations in RalA to disrupted one interaction but retain the other [26, 27], thus creating mutants that only mobilize one of two effectors in the exocyst. To this end, we introduced an E38R substitution into active RalA (G23V) to uncouple it from Sec5, or a K47E mutation that couples Exo84 from active RalA, based on the structural and biochemical work reported recently [26, 27]. Both forms of RalA uncoupling mutants showed little difference in activity state *in vivo*, as confirmed by interaction with GST-RalBP1 RBD (Ral-binding domain) in a pulldown assay (Figure 4.2B). As expected, RalAG23V/E38R was selectively uncoupled from GST-Sec5 RBD, while RalAG23V/K47R failed to interact with only GST-Exo84 RBD (Figure 4.2B).

We introduced GFP control, active RalA, and these two uncoupling mutants of active RalA into adipocytes by lentiviral infection, and then assayed glucose uptake. Intriguingly, while active RalA was able to promote glucose transport as shown above, loss of either Sec5 binding or Exo84 binding abolished this effect of active RalA (Figure 4.2C). Taken together with the fact that knockdown of either Sec5 or Exo84 inhibited insulin-stimulated glucose transport [28], the data suggested that these two RalA effectors both serve required and non-redundant roles in organizing the exocyst.

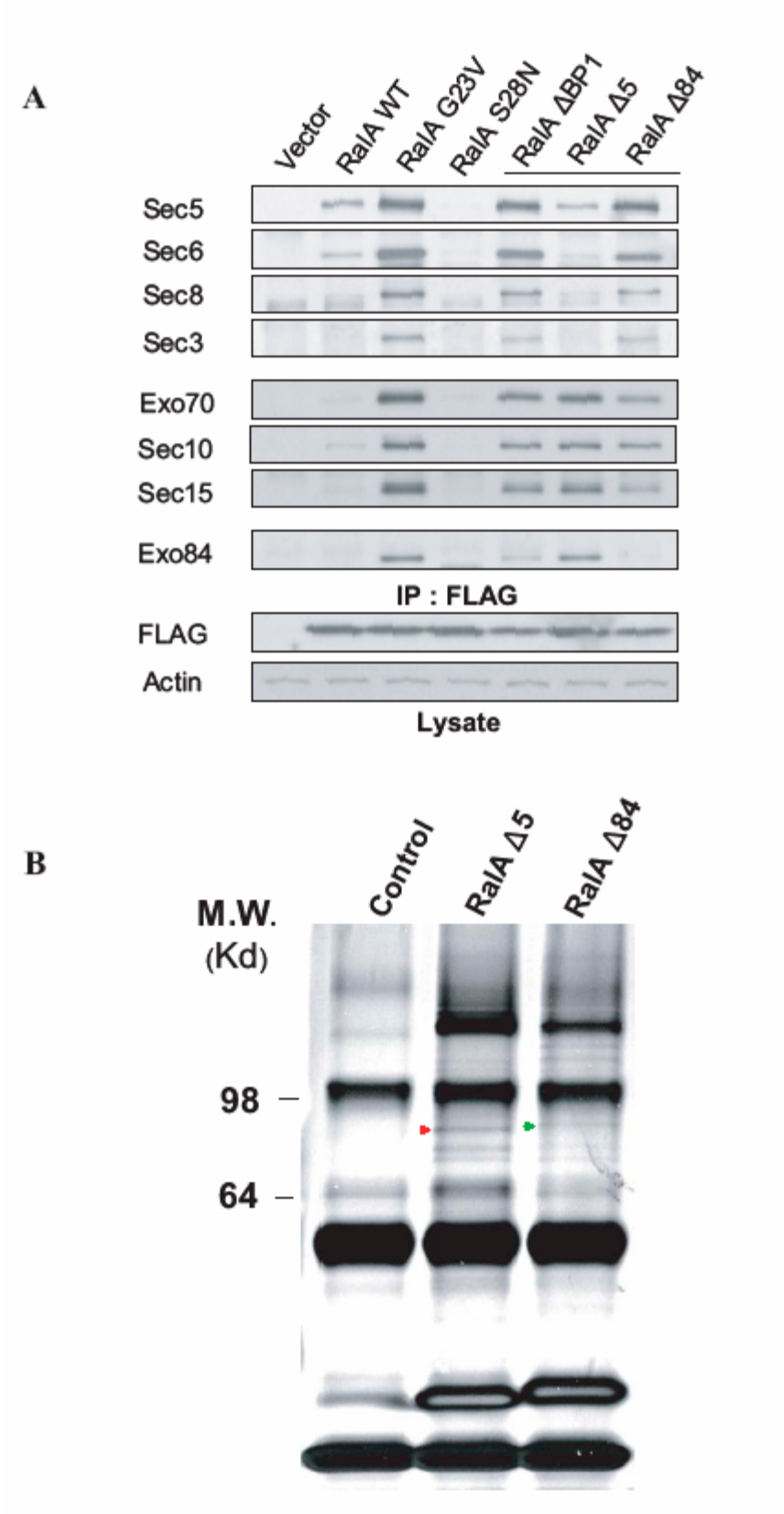
Sec5 and Exo84 belong to different branches of the mammalian exocyst with overlapping subunits. The fact that Sec5 and Exo84 are non-redundant effectors of RalA in regulating exocyst function raised the possibility that these two subunits may

Figure 4.2 Both Exo84 and Sec5 are required for exocyst function. (A) Structural illustration of the interaction interfaces between Sec5 RBD and active RalA, or Exo84 RBD and active RalA, respectively. Structures are derived from previous X-ray crystallographic studies [26, 27]. Δ5, RalA G23VE38R that does not interact with Sec5 due to collision with R27 in Sec5; Δ84 RalA G23VK47E that does not interact with Exo84 due to collision with E269 in Exo84. **(B)** Verification of the RalA uncoupling mutants by effector domain pull down. Cos-1 cells were transfected with constructs that express the indicated RalA mutants and lysed. Cell lysates were subjected to pulldown experiments with Ral-binding-domains (RBDs) from RalBP1, Sec5, or Exo84. **(C)** Both Exo84 and Sec5 are required for promoting glucose uptake by RalA. 3T3-L1 adipocytes infected with lentiviruses expressing the indicated proteins were subjected to 2-DG uptake assay. Left, glucose uptake in the basal states; right, glucose uptake after insulin stimulation. Asterisk, $p<0.05$; double asterisk, $p<0.001$. Experiment shown is representative of two independent experiments.


A
Δ5:
RalA E38R
Sec5 R27
Sec5 RBD
RalA
Exo84 RBD
RalA
Δ84:
RalA K47E
Exo84 E269
B
RalA G23V
RalA Δ84
RalA Δ5
Sec5 RBD
Exo84 RBD
RalBP1 RBD
Pulldown
Lysate
C
Basal Glucose Uptake
40
30
20
10
0
**
GFP
Active RalA
RalA Δ5
RalA Δ84
Insulin-Stimulated Glucose Uptake
150
140
130
120
110
100
90
80
*
GFP
Active RalA
RalA Δ5
RalA Δ84

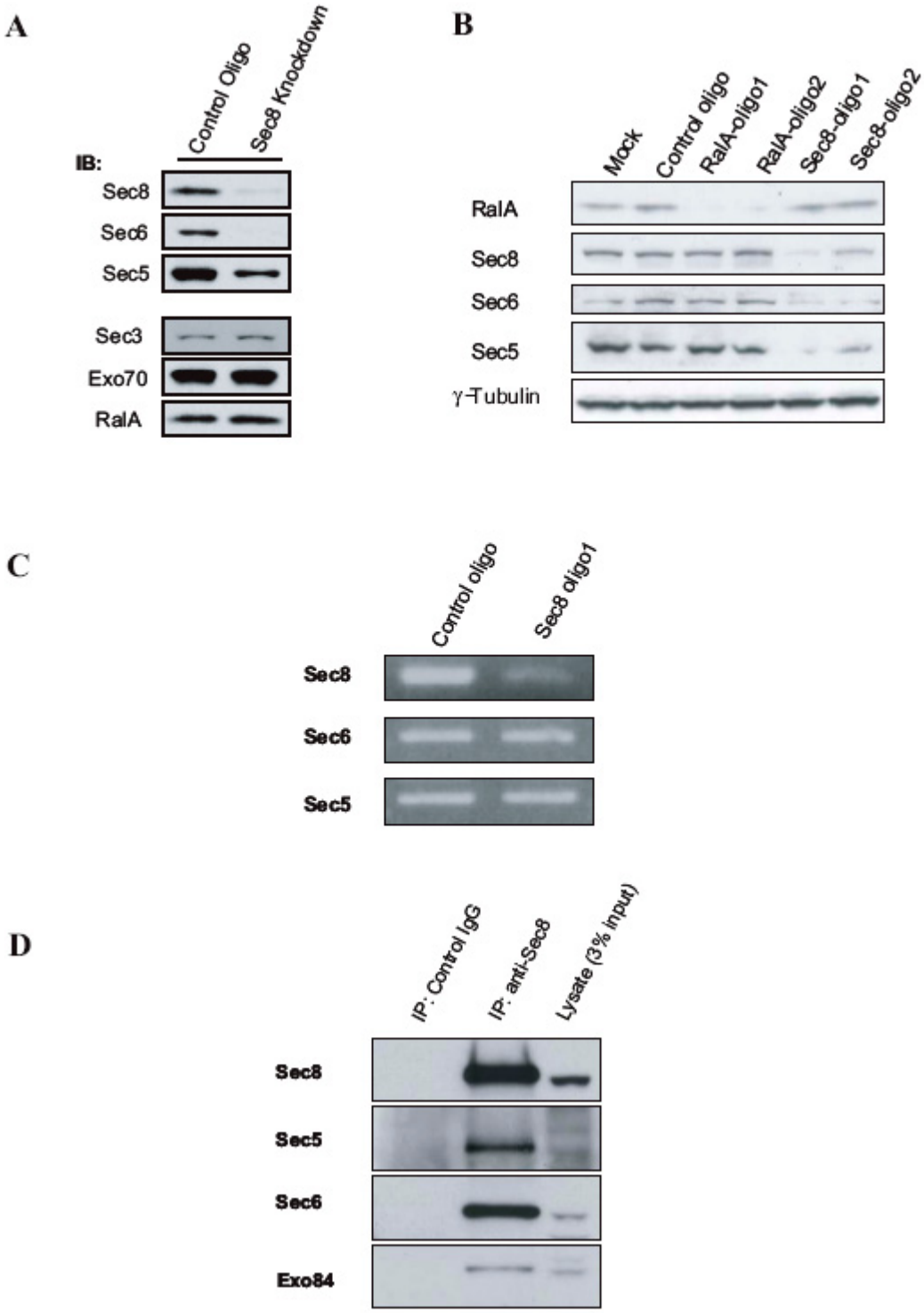
belong to distinct sub-complexes that assemble into a holo-complex. However, this hypothesis was confounded by the observation that all the subunits of the mammalian exocyst could be co-purified as a single stable complex by chromatography or sucrose gradient [31]. We took a different approach to test whether the exocyst has different pools of sub-complexes, reasoning that if the exocyst always exists in a single complex, then either of the uncoupling mutants should still bring down the entire complex in a fashion similar to wild type RalA. Otherwise, these RalA mutants would interact with different groups of exocyst subunits, assuming the existence of different sub-complexes. We thus immuno-precipitated FLAG-tagged RalA constructs that were transfected into Cos cells, followed by western blotting to detect exocyst proteins (Figure 4.3A). As expected, the exocyst proteins co-precipitated with RalA in a GTP-dependent manner, and RalAG23V/E38R or RalAG23V/K47E failed to precipitate Sec5 or Exo84, respectively. This indicated that the exocyst exists in at least two branches that contain either Sec5 or Exo84. Furthermore, the data also suggested that the branch containing Exo84 also contained Sec10, Sec15, and Exo70. Unexpectedly, while the Sec5-containing branch included Sec6, Sec8, and Sec3, which were excluded from the Exo84-containing branch, it also consisted of three overlapping subunits with the latter branch, including Sec10, Sec15, and Exo70 (Figure 4.3A). Although we cannot completely rule out the presence of Exo84 in the Sec5-containing branch, we reasoned that this branch may only contain a small amount of Exo84, which could be confirmed by silver staining of the proteins associated with the two uncoupling mutants after IP (Figure 4.3B).

Figure 4.3 Sec5 and Exo84 belong to different branches of exocyst sub-complexes with overlapping subunits. (A) Different but overlapping exocyst subunits interact with RalA uncoupling mutants. Cos-1 cells expressing the indicated FLAG-tagged RalA mutants were subjected to anti-FLAG IP and western blotting with the indicated proteins. **(B)** Exo84 is largely missing from the exocyst branch associating with the RalA Exo84-uncoupling mutant. Silver staining of the associated proteins with indicated RalA mutants after anti-FLAG IP and SDS-PAGE. Red arrow: Exo84 (molecular weight: ~84 Kd); green arrow: Sec6 (Molecular weight: ~87 Kd).


A
Vector
RalA WT
RalA G23V
RalA S28N
RalA ΔBP1
RalA Δ5
RalA Δ84
Sec5
Sec6
Sec8
Sec3
Exo70
Sec10
Sec15
Exo84
IP : FLAG
FLAG
Actin
Lysate
B
Control
RalA Δ5
RalA Δ84
M.W.
(Kd)
98
64

Sec8, Sec6, and Sec5 form a sub-complex. The overlapping subunits in the two branches of the exocyst indicated these may serve as intermediates for the assembly of the holo-complex during vesicle targeting to the plasma membrane. Indeed, Exo84, along with Sec10 and Sec15, were reported to adapt a vesicular localization [25]. We thus focused on the set of subunits that were unique in the two branches of the complex, including Sec8, Sec6, Sec5 and Sec3, reasoning that these might link the exocyst to the plasma membrane. Knock down of Sec8 in 3T3-L1 adipocytes cells by siRNA led to decreased levels of Sec6 and Sec5 (Figure 4.4 A), but not Sec3, Exo70, or any of the other exocyst proteins (data not shown). Similar effects on Sec5 and Sec6 were observed with Sec8 knockdown using two additional siRNA oligos in Hela cells of human origin, but not with control oligos or two oligos against RalA (Figure 4.4B), suggesting that this phenomenon was not due to non-specific effects of RNAi. Furthermore, unlike Sec8, mRNA levels of Sec6 or Sec5 remained unchanged when transfected with a siRNA oligo against Sec8 (Figure 4.4C), confirming that the depletion of Sec6 or Sec5 induced by knock down of Sec8 is due to post-translational protein degradation rather than loss of mRNA. This effect is reminiscent of cases in which complex subunits undergo degradation when their interacting proteins are depleted by knockout or knockdown [32, 33], and indicated that Sec5, 6, and 8 assembled into a sub-complex via direct interactions. Indeed, these three proteins associated tightly with each other in a co-immunoprecipitation experiment (Figure 4.4D), compared with the other exocyst subunit Exo84. Taken together, these data suggest that Sec8, Sec6 and Sec5 form a stable sub-complex.

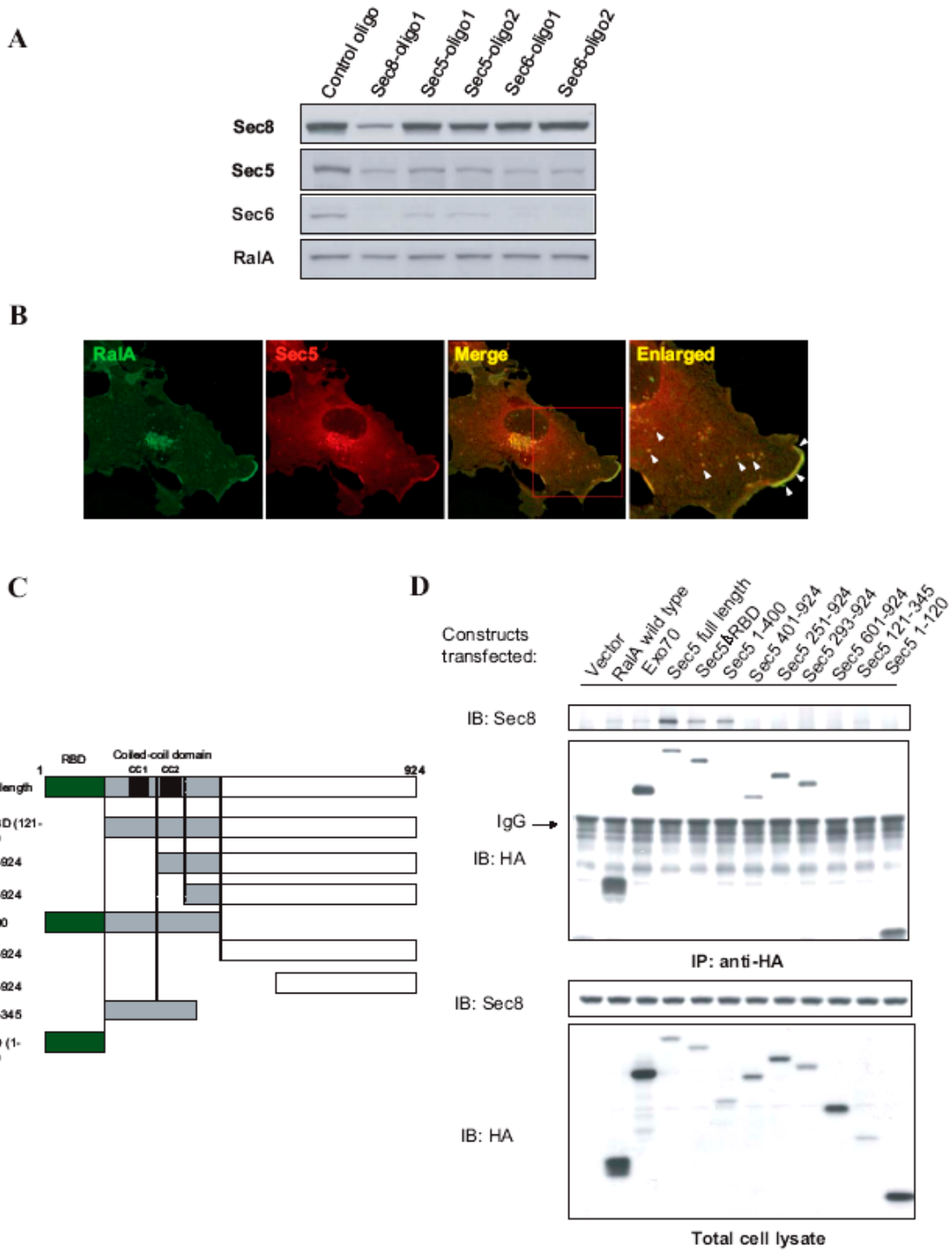
Figure 4.4 The exocyst subunits Sec8, Sec6, and Sec5 form a sub-complex independent of Ral activity. (A) Knockdown of Sec8 leads to depletion of Sec5 and Sec6, but not other exocyst proteins. 3T3-L1 adipocytes were transfected with 100 pmols of the indicated siRNA oligos and lysed in SDS containing buffer. Cellular protein levels were determined by western blotting following SDS-PAGE. **(B)** Knockdown of Sec8, but not RalA, leads to depletion of Sec6 and Sec5 in cells of human origin. Hela cells were transfected with 100 nM of the indicated siRNA oligos and lysed in SDS contained buffer. Cellular protein levels were determined by western blotting following SDS-PAGE. **(C)** Knockdown of Sec8 does not affect the mRNA levels of Sec6 or Sec5. Total RNA of Hela cells transfected with the indicated oligos were subjected to RT-PCR with specific primers for Sec8, Sec6, and Sec5. **(D)** Sec8 IP enriches Sec6 and Sec5, but not Exo84. Lysates of Cos-1 cells were immuno-precipitated using control or Sec8 antibody. IPs and total cell lysates were subjected to SDS-PAGE and western blot.


A
Control Oligo
Sec8 Knockdown
IB:
Sec8
Sec6
Sec5
Sec3
Exo70
RalA
B
Mock
Control oligo
RalA-oligo1
RalA-oligo2
Sec8-oligo1
Sec8-oligo2
RalA
Sec8
Sec6
Sec5
γ-Tubulin
C
Control oligo
Sec8 oligo1
Sec8
Sec6
Sec5
D
IP: Control IgG
IP: anti-Sec8
Lysate (3% input)
Sec8
Sec5
Sec6
Exo84

Sec8 controls the assembly of the Sec5/6/8 sub-complex Studies in yeast and flies have suggested that the exocyst builds up the vesicle:plasma membrane connection via subunit interactions that occur at different stages of vesicle targeting, with some subunits directing the localization of others [8, 14]. In this regard, we tested whether a hierarchy may also exist in the Sec5/6/8 complex. Interestingly, while knockdown of Sec8 dramatically reduced Sec5 and Sec6, knockdown of Sec5 or Sec6 had no effect on cellular Sec8 levels, but led to decreased expression of each other (Figure 4.5A). These data suggest a hierarchy in which Sec8 may regulate the localization of Sec6 and 5, the latter of which recognizes RalA. Consistent with this notion, we found that Sec5 co-localized with RalA both on the plasma membrane and the vesicular structures (Figure 4.5B). On the other hand, knockdown of RalA was without effect on levels of Sec5, Sec6, or Sec8 protein, suggesting that the assembly of this sub-complex is independent of RalA. To further confirm this finding, we generated a series of Sec5 mutants (Figure 4.5C), and tested their interaction with endogenous Sec8. Mutant forms of Sec5 lacking the Ral binding domain, but not those lacking the coiled-coil domains, still interacted with Sec8 (Figure 4.5E). In addition, we found that active mutants of RalA (G23V or F39L) were without out effect on the formation of a Sec5/Sec6/Sec8 sub-complex (data not shown). Taken together, these data supported the idea that Sec5, Sec6 and Sec8 pre-assemble into a sub-complex under the control of Sec8, possibly targeting RalA-localized vesicles to the plasma membrane.

Sec5 bridges RalA to Sec8. Sec8 has been shown to associate with SAP97 [6], a scaffolding protein on the plasma membrane, via its C-terminal PDZ domain binding

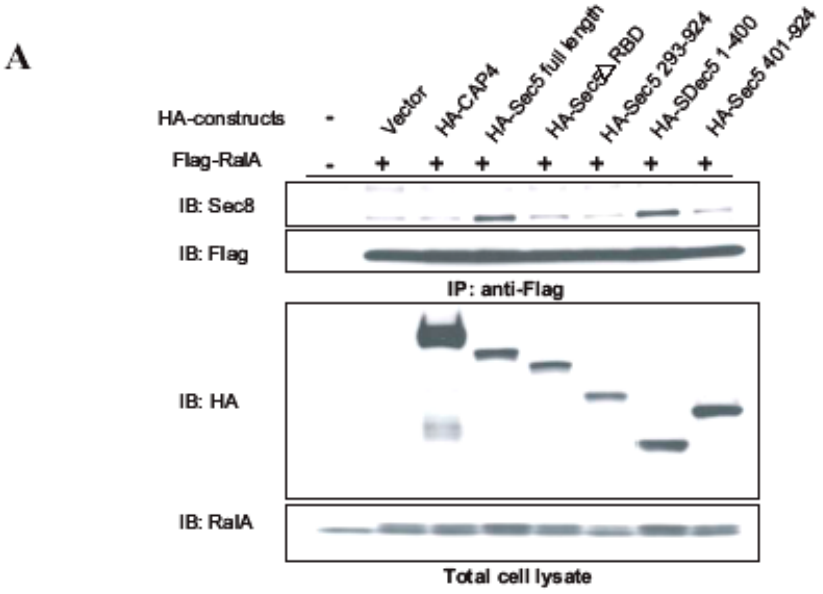
Figure 4.5 Assembly of the Sec5/Sec6/Sec8 complex under the control of Sec8 but not RalA (A) Sec8 regulates the stability of Sec5, Sec6 protein. Hela cells were transfected with the indicated siRNA oligos, and lysed in SDS-containing buffer. Cell lysates were subjected to SDS-PAGE, followed by western blotting against the indicated proteins. **(B)** Sec5 co-localizes with RalA on both plasma membrane and intracellular vesicular structures. Cos-1 cells transfected with eGFP-RalA (green) and full length HA-Sec5 (red) were stained with HA antibody. **(C)** Schematic figures of Sec5 constructs used. All constructs were HA-tagged. **(D)** RalA-independent interaction between Sec8 and Sec5. Cos-1 cells were transfected with indicated constructs, lysed, and subjected to anti-HA IP. IPs and cell lysates were subjected to SDS-PAGE and western blotting using Sec8 and HA antibodies.

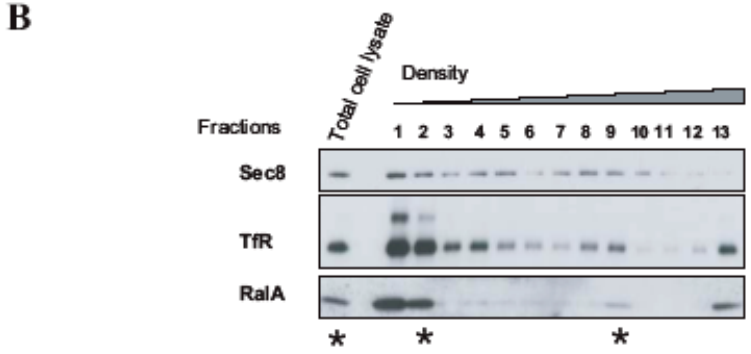

A
Control oligo
Sec8-oligo1
Sec5-oligo1
Sec5-oligo2
Sec6-oligo1
Sec6-oligo2
Sec8
Sec5
Sec6
RalA
B
RalA
Sec5
Merge
Enlarged
C
RBD
Coiled-coil domain
cc1
cc2
1
924
Full length
ΔRBD (121-924)
251-924
293-924
1-400
401-924
601-924
121-345
RBD (1-120)
D
Constructs transfected:
Vector
RalA wild type
Exo70
Sec5 full length
Sec5ΔRBD
Sec5 1-400
Sec5 401-924
Sec5 251-924
Sec5 293-924
Sec5 601-924
Sec5 121-345
Sec5 1-120
IB: Sec8
IgG
IB: HA
IP: anti-HA
IB: Sec8
IB: HA
Total cell lysate

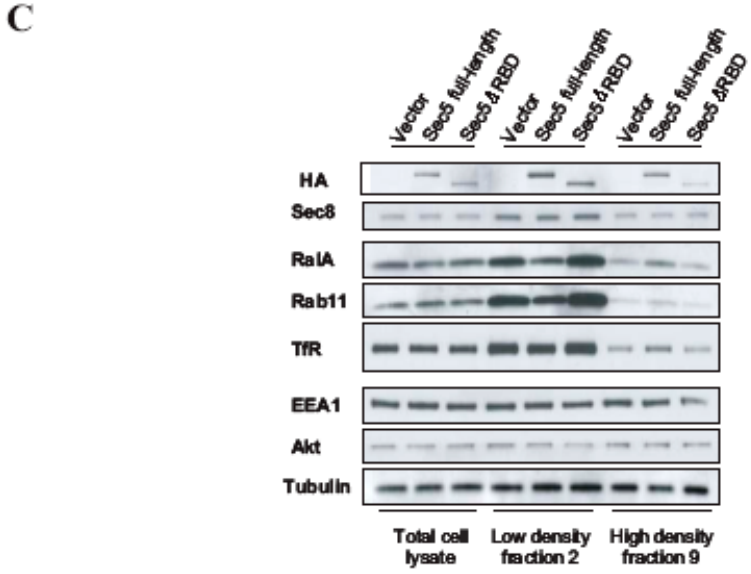
motif. This promoted us to further test whether Sec5 may bridge RalA to Sec8, as a direct mechanism by which RalA vesicles are targeted to the plasma membrane. When assessed by co-IP, the interaction between wild type RalA and endogenous Sec8 was enhanced by modest over-expression of full length Sec5 or Sec5 1-400, but not Sec5 mutants lacking the Ral binding domain (Figure 4.6A). Interestingly, the Sec5 ΔRBD mutant, which can interact with endogenous Sec8 but not RalA, failed to inhibit the interaction between RalA and Sec8. This data, together with the gain-of-interaction trait observed with wild type Sec5, suggest that Sec8 may exist in monomer in molar excess to Sec5, which in turn connects to RalA vesicles. We further tested whether Sec5 may modulate the distribution of some RE proteins via its interaction with RalA. A non-linear opti-prep gradient was generated to separate different membrane compartments based on their density in quiescent Cos cells. A fraction of RalA was concentrated in a high density fraction that is enriched with both the TfR and Sec8 (Figure 4.6B). Expression of wild type Sec5, but not Sec5ΔRBD, produced the enrichment of RalA in this fraction, accompanied by the movement of Rab11 and TfR, but not EEA1, Akt, or Sec8 (Figure 4.6C), suggesting a selective effect of Sec5 on RE membranes where RalA resides.

Sec3 potentially links the exocyst to the SNARE complex The idea Sec5, Sec6, and Sec8 exist in a sub-complex led us to investigate the potential role of Sec3, the remaining non-overlapping subunit in the two branches of the exocyst. When submitted to a BLAST search, we found that the N-terminus of Sec3 protein shared significant homology with Syntaxin-binding-protein 6 (Stxbp6) (Figure 4.7A), a protein previously shown to interact with Syntaxin1 [34]. Hence, it remains highly possibly that Sec3 represent a potential

Figure 4.6 Sec5 bridges RalA to Sec8. (A) Sec5 promotes the interaction between RalA and Sec8. Cos-1 cells were transfected with indicated constructs, lysed, and subjected to anti-FLAG IP. IPs and cell lysates were subjected to SDS-PAGE and western blotting using the indicated antibodies. **(B)** Separation of low-density and high density membranes on a non-linear gradient. Post nuclear supernatant of Cos-1 cell homogenate was mixed 1:1 with opti-prep, and spun at 350,000g for 1 hour. Separation of membranes is determined by western blotting against Sec8, TfR, and RalA. **(C)** Sec5, but not Sec5ΔRBD redistributes RE proteins including RalA, Rab11, and TfR. Cos-1 cells were transfected with empty vector, Sec5 full length, or Sec5ΔRBD, and then fractionated according to what was described in (B). Total cell lysate, low density fraction (fraction 2), and high density fraction (fraction 9) of each gradient were subjected to SDS-PAGE and western blotting against the indicated antibodies.


A
HA-constructs
-
Vector
HA-CAP4
HA-Sec5 full length
HA-Sec5ΔRBD
HA-Sec5 293-924
HA-SDec5 1-400
HA-Sec5 401-924
Flag-RalA
- + + + + + + +
IB: Sec8
IB: Flag
IP: anti-Flag
IB: HA
IB: RalA
Total cell lysate


B
Total cell lysate
Density
Fractions
1 2 3 4 5 6 7 8 9 10 11 12 13
Sec8
TfR
RalA
* * *


C
Vector
Sec5 full-length
Sec5 ΔRBD
Vector
Sec5 full-length
Sec5 ΔRBD
Vector
Sec5 full-length
Sec5 ΔRBD
HA
Sec8
RalA
Rab11
TfR
EEA1
Akt
Tubulin
Total cell lysate
Low density fraction 2
High density fraction 9

link between the vesicle tethering complex exocyst and the vesicle fusion complex SNARE (Figure 4.7B).

Discussion

Insulin-stimulated glucose transport is a key physiological event that requires tight regulation to ensure the specificity and facilitate the efficiency of the process [2, 35]. In this regard, the trafficking of the insulin-responsive glucose transporter Glut4 is controlled by transport machineries that are governed by insulin [3, 36]. The exocyst represents a unique point of integration, by responding to the activation of TC10 and RalA [5, 28], two small GTPases regulated by insulin. However, the mechanisms underlining exocyst function remain unclear, as are the means by which GTPases regulate the dynamics of the complex [25]. We demonstrate here that RalA regulates the function of the exocyst in fat cells via both of its effectors, Sec5 and Exo84. Furthermore, the complex appears to be categorized into two branches containing either Sec5 or Exo84, but with overlapping subunits including Sec10, Sec15, and Exo70.

The exact mechanisms by which the mammalian exocyst assembles into a functional complex remain controversial [31]. Our data supported the presence of sub-complexes of the exocyst. Intriguingly, we showed that the different pools of the complex may contain overlapping subunits, suggesting that these may represent intermediates that bridge different pools of the exocyst during the assembly of the holo-complex. Furthermore, Sec5, 6, and 8 assemble into a stable sub-complex under the control of Sec8, while Sec5

A

```
Sec3 N      KHALQRDIFTPNDERLLSIVNVCKAGKKKKN----------CFLCATVTTERPVQVKVV  53
            K A+ ++IF P DER+L  + V +  KKK              ++C +VT ++P Q  +
Stxbp6      KSAISKEIFAPLDERMLGAIQVKRRTKKKIPFLATGGQGEYLTYICLSVTNKKPTQASIT  63

Sec3 N      KVKKSDKGDFYKRQIAWALRDLAVVDAKDAIKENPEFDLHFEKVY-KWVASSTAEKNAFI  112
            KVK+ +    + R+  W L  L  V+  D  +++ EFDL FE  + +WVAS+ +EK  F
Stxbp6      KVKQFEGSTSFVRRSQWMLEQLRQVNGIDPNRDSAEFDLLFENAFDQWVASTASEKCTFF  123

Sec3 N      SCIWKLNQRYLR-KKIDFVNVSSQLL-EESVPSGENQSVAGGDE---EAVDEYQELNARE  167
              +   QRYL  +K +F+N  S+++   S+      SV    +   +A++E  E   R
Stxbp6      QILHHTCQRYLTDRKPEFINCQSKIMGGNSILHSAADSVTSAVQKASQALNERGERLGRA  183

Sec3 N      EQDIEIMMEGCECAISNAEAFAEK  191
            E+  E M    +     A   A K
Stxbp6      EEKTEDMKNSAQQFAETAHKLAMK  207
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B

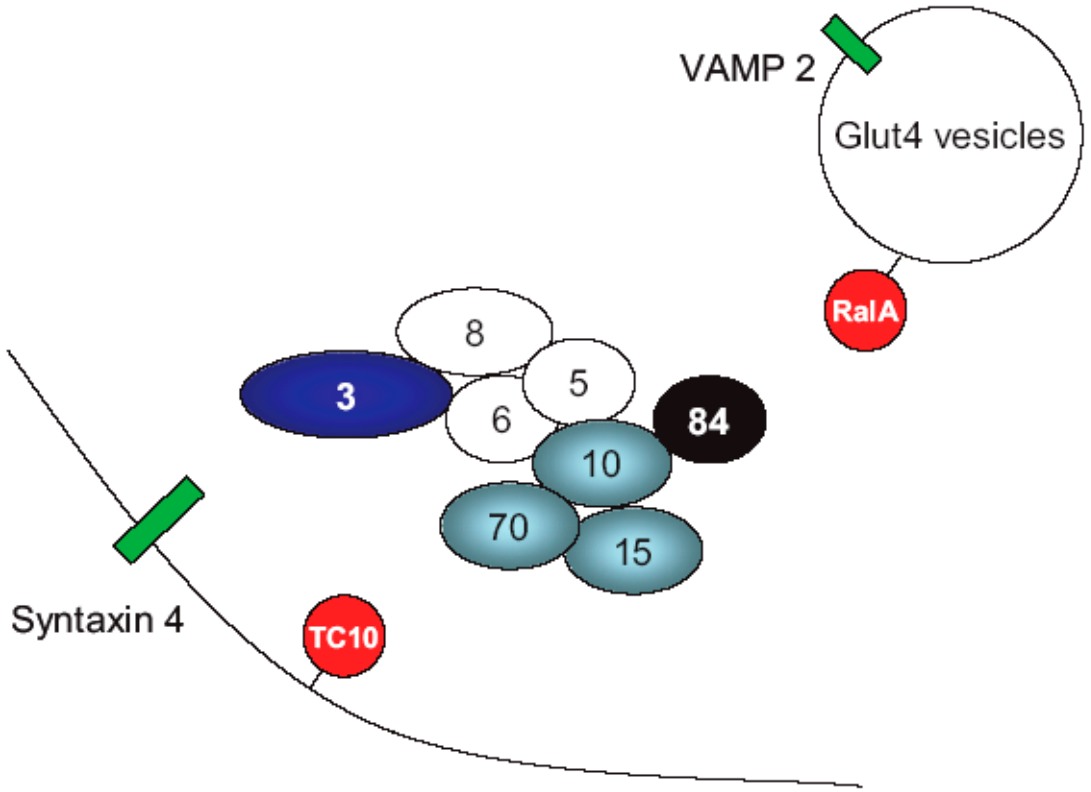

Figure 4.7 Sec3 potentially links the exocyst to the SNARE complex. (a) Alignments of the N-terminal domain of mouse Sec3 (Sec3 N) and Syntaxin-binding protein6 (Stxbp6). (b) A proposed model for the architecture of the mammalian exocyst complex.

bridges RalA-localized membranes to Sec8. It is noteworthy that Sec8 has been shown to associate with a scaffolding protein of the MAGUK family on the plasma membrane [6, 37]. Thus, the Sec5/6/8 sub-complex may directly contribute to the targeting of exocytic vesicles to their target sites on the plasma membrane. After being tethered by exocyst complex to target sites on the plasma membrane, the exocytic vesicles eventually undergo membrane fusion catalyzed by the SNARE complex [9, 38]. Over-expression of several exocyst subunits in adipocytes resulted in enhanced exocytosis of Glut4 [5, 7, 15]; a process that requires the action of SNARE proteins [2]. Thus, it is tempting to speculate that the function of the exocyst and the SNARE complex are coupled in some way. Though this has not been proven in higher organisms, studies in yeast have shown that the exocyst genetically and biochemically interacts with the SNARE proteins and their regulators [39]. Our data suggest that Sec3 could be the most far-reaching subunit in the complex towards the plasma membrane, making it an ideal candidate for connecting with t-SNARE proteins. Hence, it will be of interest to investigate whether Sec3 interacts with Syntaxin4 and modulates its function in the context of regulated exocytosis of Glut4.

Materials and Methods:

Constructs. Full length Sec5 was obtained from a 3T3-L1 cDNA library by PCR, completely sequenced, and then cloned into a pKH3 vector [5]. Truncated mutants of Sec5 were generated by PCR and then cloned into a pKH3 vector. Sec5 RBD-(1-120) was cloned into peYFP-C1 vector (BD biosciences). RalBP1 RBD (397-519) was also cloned into pGEX-4T2 vector (Pharmacia). RalA was cloned into a pK-FLAG vector or a peGFP-C3 vector (BD biosciences). Point mutations of RalA were generated using

Quickchange Site-Directed Mutagenesis kit from Stratagene, and completely sequenced at the University of Michigan Sequencing Core.

Cell culture, transfection, and reagents. Cos-1 and HeLa cells were grown in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal bovine serum and 100 Unit/ml streptomycin (Invitrogen). Maintain and differentiation of 3T3-L1 pre-adipocytes were carried out as previously reported [40]. Cos-1 cells were transfected using Fugene 6 (Roche Applied Biosciences) according to the manufacturer's directions. All chemicals and hormones were from Sigma.

Immunofluorescence and antibodies. Cells were grown on glass coverslips and washed with PBS before fixation. After fixation with -20^{o}C methanol for 3 minutes, cells were re-hydrated in PBS, and then blocked with 1% BSA and 1% chicken albumin. Primary antibodies used were α-6 integrin (1:200, rat mAb), Caveolin 1 (1:500, rabbit IgG) from BD biosciences; HA (1:500, mAb or rabbit IgG) from Santa Cruz biotechnology. Alexa-Fluor conjugated goat anti-mouse/rabbit secondary antibodies, Alexa-Fluor conjugated Phalloidin and Vector Shield mounting medium were from Molecular Probe. The following antibodies were used in biochemical assays: RalA and EEA1 mAbs were from BD biosciences; tubulin mAb was from Sigma; Sec6 mAb was from Stressgen; rabbit anti-Exo84 was from Orbigen; anti-Sec5 mAb has been described before [41].

Immunoprecipitation and Western blot. Cells were washed with PBS and lysed for 30 minutes at 4^{o}C in buffer (buffer A) containing 100mM Tris (pH 7.5), 1% NP-40, 135 mM

NaCl, 1 mM EDTA, 1.0 mM sodium orthovanadate, 10 mM NaF, and protease inhibitor tablets (Roche Applied Biosciences). Cell lysates were incubated with 10-15 μl M2 anti-FLAG beads (Sigma) or 4-8 μg of indicated antibodies for 2-4 hours at 4^0C before protein A/G agarose (Santa Cruz Biotechnology) was added for additional 2 hours. After washing with lysis buffer 3 times, beads were boiled in SDS-PAGE sample buffer. The eluates were subjected to SDS-PAGE and transferred to nitrocellulose. Individual proteins were detected with specific antibodies and visualized by blotting with HRP-conjugated secondary antibodies.

Opti-prep gradient. Cos cells were washed with PBS, homogenized in HES buffer (20mM Hepes pH 7.4, 1mM EDTA, 250 mM Sucrose) 10 times with a ball bearing homogenizer (Wheaton), and spun at 3,000g for 3 minutes to generate post-nuclear supernatant (PNS). The discontinuous gradient was generated by spinning PNS in 30% iodixanol at 72,000 rpm in a NVT90 rotor for 1 hour at 4^oC, and then fractionated into 13 fractions. To compare RalA distribution among different gradients, total input, same volume of faction 2 and fraction 9 of each gradient were loaded onto the same gel and subjected to WB.

siRNA sequence and knockdown. siRNA oligos were ordered from Invitrogen. The following siRNA oligos against human nucleotides sequence were used. RalA: 5'-CCAAGGGUCAGAAUUCUUU-3' (oligo-1 sense sequence), 5'-GCUAAUGUUGACAAGGUAU-3' (oligo-2 sense sequence); Sec8: 5'-GCU UUC UCC AAU CUU UCU A-3' (oligo-1 sense sequence), 5'-CCU UGA UAC CUC UCA CUA U-

3’ (oligo-2 sense sequence); Sec6 5’-CCA GAU GUU UGA ACA GAA U-3’ (oligo-1 sense sequence), 5’-GGA AGA CUA UUU CAA CGAU-3’ (oligo-2 sense sequence); Sec5: 5’-CCA AAU GAA GGG AUA CCA U-3’ (oligo-1 sense sequence), 5’-CCA ACA CAA GUG GAU CCU U-3’ (oligo-2 sense sequence). Stealth oligos against mouse Sec8 nucleotides sequences and control oligos were also from Invitrogen and have been published previously [40]. 100 nM oligos were transfected into HeLa cells using oligofectamine according to the manufactory’s instruction. SiRNA knockdown in 3T3-L1 adipocytes was performed as previously described. Cells were either harvested in SDS-PAGE sample buffer for Western blot or processed for microscopy.

RT-PCR Total mRNA was extracted using RNeasy Mini kit from Qiagen according to the manufacturer’s instructions and normalized. RT reaction was carried out using reverse transcriptase from Invitrogen. The following DNA oligos were used for PCR. Sec8 (2523): 5- CGA AGG CCT GGG CCA CCT GA-3’, Sec8 (end): 5’-CTA AAC GGT AGT TAT CTT CT-3’; Sec6 (start): 5’- ATG AAG GAG ACA GAC CGG GA-3’, Sec6 (520): 5’-CGT GCT GCC AAA GTA GCC AT-3’; Sec5 (start): 5’-ATG TCT CGA TCA CGA CAA CC-3’, Sec5 (360): 5’-CCT GTC AGT GCG CAT ATC AT-3’.

Lentiviral work and glucose uptake- Lentiviral expression system in adipocytes has been described before [42]. Adipocytes were first infected with lentivirus in the presence of 8μg/ml polybrene, and subjected to biochemical assays or glucose uptake after 3-4 days. Insulin-stimulated 2-Deoxyglucose uptake assay was performed as previously reported [40]. Glucose incorporation was determined after normalizing with protein concentration measured by the Bradford assay. Standard t-test (2-tailed) was performed to analyze the data. For 2-DG uptake assays, error bars represented standard deviation mean from three replicated samples for each condition tested. Error bars elsewhere represented standard deviation from at least three independent experiments.

References:

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Chapter 5

Conclusions and Perspectives

Insulin is the master regulator of glucose and lipid homeostasis. One of its primary actions is to stimulate the transport of glucose into fat and muscle cells, a process mediated by the glucose transporter Glut4. Insulin increases glucose uptake by stimulating the translocation of Glut4 from intracellular storage sites to the plasma membrane [1-3]. Defects in this action of insulin represent the first lesions in the development of type 2 diabetes, as well as being a primary symptom of the disease [3, 4]. In this regard, delineation of the exact mechanisms underlying insulin-stimulated Glut4 trafficking will be crucial to developing new approaches for the prevention and treatment of diabetes and related disorders. Furthermore, the trafficking of Glut4 is one of the best models of hormone-regulated endocytic recycling, thus potentially shedding light on similar transport processes in many physiological scenarios [5, 6].

One of the key traits of Glut4 trafficking is its robust insulin responsiveness [2]. To maximize the efficiency of this process, multiple transport machineries are mobilized upon the activation of insulin signaling pathways, which ensure the specificity of insulin action, and permit numerous opportunities for crosstalk from other pathways designed to modulate insulin action [7, 8]. One such example is the exocyst [9, 10], an

evolutionarily conserved vesicle tethering complex that works under the dual control of TC10 [9] and RalA [11], two GTPases activated by insulin signaling.

The exocyst is comprised of eight subunits, including Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 [12]. Studies in yeast have revealed that these proteins assemble into a long targeting patch between exocytic vesicles and their target membrane, governed by direct interactions with at least two different GTPases anchored on either compartment [13]. Thus, the exocyst has been proposed to mediate the initial recognition between vesicle and plasma membrane with flexibility and reversibility, in a manner determined by the activity states of the GTPases [12]. Furthermore, the exocyst has been shown to co-operate with molecular motors as well as the SNARE proteins and their regulators in yeast [13]. In this regard, the exocyst may also contribute to the velocity of transport and facilitate the final fusion with the plasma membrane, thus serve as an integrative component during transport to the plasma membrane.

Despite adopting a similar composition as its yeast counterpart, the exocyst in higher organisms shows significant differences. Firstly, the biological function of the exocyst appears to be more specialized [14], although its physiological functions remain to be fully established in specific settings. Secondly, the complex responds to a different set of GTPases, while their actions in exocyst-mediated transport have not been fully elucidated [15]. Thirdly, the organization of the complex appears to adapt to the different regulatory mechanisms [16]. Furthermore, whether or how the exocyst coordinates with other transport machineries in higher organisms is largely unknown. Work presented in this

thesis aims to fill some of the gaps in the knowledge highlighted above, primarily by focusing on the mammalian exocyst and the GTPase RalA.

Chapter 2 of the thesis described the initial work involved in the characterization of the roles of RalA and the exocyst in vesicle transport. RalA localizes to the endocytic structures surrounding the centrosome, and re-localizes to cytokinetic structures during the cell cycle, as does the exocyst. Disruption of the function of either RalA or the exocyst leads to similar blockades in the final separation of two dividing cells during cytokinesis, possibly due to defects in vesicle transport to the sites of abscission. While the data suggest a functional connection between RalA and exocyst, further attempts to dissect this process will be challenging, largely due to the promiscuity of trafficking routes involved in cell division and the effects secondary to cell cycle arrest.

Studies in Chapter 3 focus on the roles of RalA and the exocyst in the regulated endocytic recycling of Glut4 in response to insulin. RalA associates with Glut4-enriched vesicular membranes, and can interact with the native exocyst complex in adipocytes. Insulin regulates the activity of RalA in a PI-3 kinase-dependent manner. Disruption of the function of RalA inhibits insulin-stimulated glucose transport. A similar effect is observed upon loss of Sec5 or Exo84, two exocyst subunits directly interacting with RalA. Furthermore, RalA associates with Myo1c, a molecular motor previously implicated in Glut4 trafficking to the plasma membrane. This association is not dependent on the activity state of RalA, but can be modulated by Calmodulin, which functions as the light chain for Myo1c during glucose transport. Disruption of the function of Myo1c and the

exocyst together leads to a synergistic inhibition of insulin action, suggesting that these proteins coordinate with each other during insulin-stimulated glucose transport. While these data indicate a scheme in which RalA may function in a manner similar to the yeast GTPase Sec4, which localizes to the exocytic vesicles and couples the function of the exocyst and motor protein, the molecular mechanisms by which RalA regulates the dynamics of the mammalian exocyst remain to be determined.

Experiments in Chapter 4 aim to analyze the architecture and assembly of the exocyst under the regulation of RalA. Constitutively active RalA promotes glucose uptake into adipocytes in both the basal and insulin-stimulated states, a process that requires both Sec5 and Exo84. However, these two subunits belong to different branches of the exocyst: the Exo84-containing branch also includes Sec10, Sec15, and Exo70, and the Sec5-containing branch consists of Sec3, Sec6, Sec8 that are excluded from the former branch, and three overlapping subunits including Sec10, Sec15, and Exo70. Analysis of the unique subunits in the Sec5 branch uncovered a sub-complex comprised of Sec5, Sec6, and Sec8. Sec8 controls the assembly and stability of this sub-complex, and Sec5 bridges RalA to Sec8. Furthermore, the remaining non-overlapping subunit Sec3 process an N-terminal domain that shows high homology to Syntaxin-binding-protein6, and thus may represent a potential link between the exocyst complex and the SNARE proteins. It will thus be interesting to test whether the N-terminal domain of Sec3 interacts with and modulates the function of Syntaxin4, the t-SNARE protein involved in plasma membrane fusion of Glut4 vesicles.

The mechanisms by which the exocyst exerts its function are only now being unraveled. A simple hypothesis, based on the fact that loss of almost every subunit in the complex leads to lethality in all the organisms tested, is that each subunit must serve non-redundant functions. Hence, it is probably safe to hypothesize that this octameric complex is functionally coupled to multiple machineries during vesicle transport. One likely candidate is the fusion-catalyzing SNARE complex. It is noteworthy that loss of exocyst function in yeast also causes decreased assembly of the cognate SNARE complex [17]. Moreover, over-expression of several exocyst subunits in adipocytes leads to enhanced Glut4 exocytosis [9, 10]; a process that requires the function of the SNARE complex. In this regard, it would be interesting to test whether and how the exocyst is linked to SNARE proteins. One possibility, as described above, is that Sec3 may directly interact with Syntaxin4 via the N-terminal domain of the former protein. Alternatively, the yeast exocyst has been shown to interact with Lgl, an evolutionarily conserved protein implicated in the function of the SNARE complex [18]. Thus, it will be of interest to test these possibilities in physiological processes such as insulin-stimulated glucose transport.

As the action of the exocyst is likely to precede that of the SNARE complex [12, 13, 17], an important implication is that the disassembly of the exocyst must also take place after the assembly of the complex, to allow the subsequent membrane fusion. Consistent with this hypothesis, blocking GTP hydrolysis of TC10 inhibits vesicle fusion, suggesting the involvement of GAP proteins that inactivate the GTPase [19]. In this regard, the interaction between RalA and the exocyst is likely to be negatively regulated by

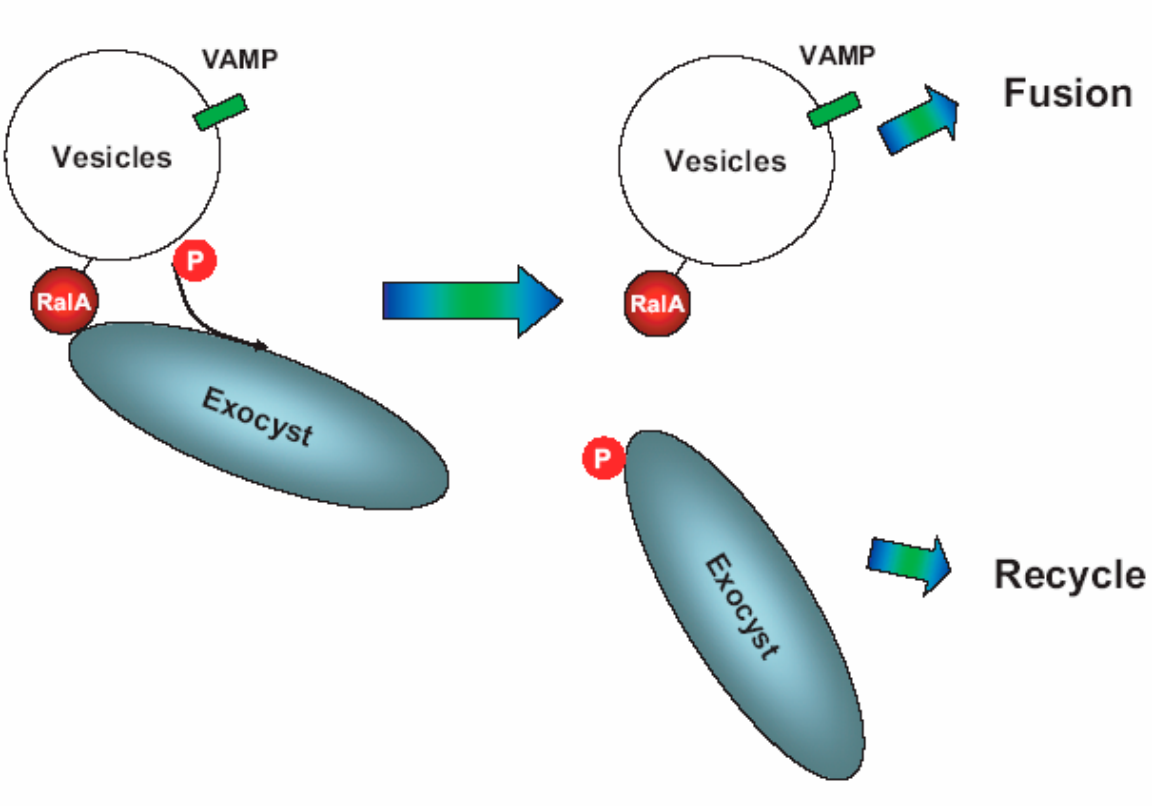

Figure 5.1 Possible mechanism that terminates RalA-exocyst interaction by phosphorylation Upon binding to RalA, Sec5 undergoes phosphorylation that disrupts the local structure of Ral-Binding-domain, thus allowing RalA vesicles for subsequent fusion. The exocyst may undergo recycling for the next round of action.

additional mechanisms, as constitutively active RalA is still able to promote glucose transport as well as the exocytosis of a few other cargo proteins. In our attempts to identify the mechanisms that regulate the disassembly of the exocyst complex, we discovered a phosphorylation event on the RalA effector Sec5. Intriguingly, binding to RalA enhances this phosphorylation on Sec5, and a mutation that mimics phosphorylation on Sec5 dramatically decreased its binding affinity with RalA (Chen and Saltiel, unpublished results). Hence, phosphorylation of the RalA effector Sec5 may represent an attractive mechanism by which the exocyst undergoes disassembly during vesicle targeting (Figure 5.1).

The mechanisms by which insulin regulates the activity state of RalA deserve further attention. One potential clue arose from the finding that the activation of RalA by insulin requires the activity of PI 3-kinase, thus implicating two possibilities. Firstly, based on the fact that the RalGPS family of RalGEFs only contain the PH and SH3 domain as regulator modules besides the GEF domain [20], it is possible that these GEF proteins may activate RalA in response to elevated PIP3 levels generated by activated PI 3-kinases. Secondly, we have recently found that both RalA activity and glucose uptake can be elevated upon depletion of a GAP protein downstream of Akt (Leto and Saltiel, unpublished observations). Thus, insulin may produce the activation of RalA by negatively regulating the function of a Ral GAP.

Despite much effort, the exact mechanisms underlying the assembly and architecture of the mammalian exocyst have only been addressed tentatively in this thesis. Studies on

this essential aspect of the complex are limited elsewhere, possibly due to its dynamic nature and a large number of possible interactions within the complex. Our data support the idea that the exocyst may assemble from different sub-complexes, and may provide clues for further investigation into the composition of the complex. In this regard, detailed structural studies may be required in the future to pave the way for understanding molecular mechanisms underlying the function of the exocyst.

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