Chapter I

INTRODUCTION

The prevalence of Diabetes Mellitus is a rising global concern. Recent statistics report that the disease had affected 347 million adults worldwide by the year 2008 [1]. An estimated 3.4 million people died from diabetes related consequences in the year 2004, and this number is projected to double between 2005 and 2030 [1]. Diabetes Mellitus is characterized by chronic hyperglycemia and dyslipidemia resulting from defects in insulin secretion, insulin action, or both. The long-term dysregulation of blood glucose and lipid contributes directly to the serious even life-threatening complications of diabetes, including nephropathy, retinopathy, neuropathy, cardiovascular disease and stroke [2].

Diabetes Mellitus can be categorized into two major classes, type 1 and type 2, based on their etiologies. Type 1 diabetes accounts for less than 10% of all cases of the disease and results from autoimmune destruction of the islet β cells of the pancreas [3]. Type 2 diabetes, which is significantly more prevalent, occurs due to a combination of reduced insulin sensitivity and production [2, 4]. The continuously worsening insulin resistance is initially compensated by over-secretion of insulin from the islet β cells, and is followed by a period of de-compensation, when the β cells eventually fail to meet the increased demand of insulin, resulting in deteriorated glucose tolerance and frank diabetes [4]. In this regard, a better understanding of the molecular mechanisms of insulin action may help in unraveling the pathogenesis of insulin resistance and in developing new therapeutic approaches for type 2 diabetes.

As the most potent anabolic hormone known, insulin promotes metabolite storage in liver, muscle and fat tissue by increasing the synthesis of glycogen, lipids and protein, while inhibiting their breakdown and release into circulation [5]. The first and rate-limiting step of energy storage in fat and muscle tissue is insulin stimulated glucose uptake, which is mediated by the insulin-responsive glucose transporter Glut4 [6]. Insulin regulates Glut4 by alternating its intracellular localization, via a regulated exocytic process that similarly occurs in a variety of physiological settings, such as controlled release of soluble factors into extracellular space, and regulated delivery of intracellular membranes for cell surface expansion [7, 8]. These features place Glut4 at the interface of two intriguing fields of biology—regulated vesicle trafficking and the insulin signaling pathway.

I. The insulin-responsive Glucose Transport Glut4

Glut4 and glucose homeostasis

In mammals, glucose homeostasis is maintained by the Glut, or Slc2, family of facilitative sugar transporters. These transporters are N-glycosylated, 12-transmembrane domain containing proteins (**Figure 1.1**) that catalyze hexose transport across cell membrane down a concentration gradient in an ATP-independent manner [9, 10]. To date, 13 different Glut proteins have been identified in human (Glut1-13), with different expression patterns and substrate specificities that reflect their distinctive physiological roles in hexose sensing and utilization [11]. Among them, Glut4 is primarily expressed in

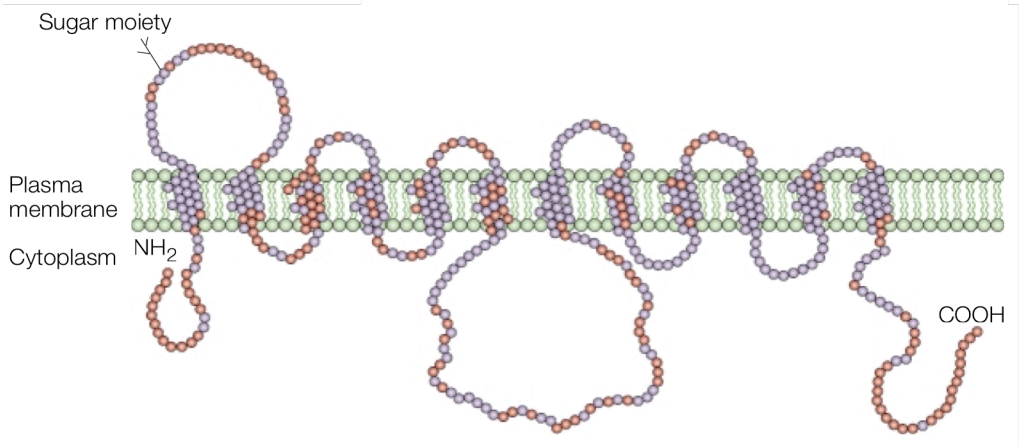


Figure 1.1 Structure of the facilitative glucose transporters Glut1-4.
The Glut family of proteins are 12 trans-membrane domain containing proteins with distinct tissue and subcellular distributions. The Glut1-4 transporters share sequential and structural homology, which are depicted in this diagram. Residues unique to Glut4 are shown in red. Modified from Bryant *et al.*, Nat Rev Mol Cell Biol, 2002. **3**(4): p. 267-77.

insulin-responsive tissues including adipose tissue and skeletal muscle and is the only known Glut family member that changes its cellular localization upon insulin stimulation [9, 12]. These characteristics render Glut4 a crucial player in maintaining glucose homeostasis in response to insulin.

The importance of Glut4 in whole body metabolism is further supported by various genetic models in which the expression of Glut4 is either ablated or enhanced. Mice with whole body Glut4 ablation (Glut4 -/-) exhibit growth retardation, cardiac hypertrophy and severely reduced adipose tissue deposits but no significant fasting or postprandial hyperglycemia, potentially due to upregulation of compensatory mechanisms that may protect them from developing frank diabetes [13, 14]. Heterozygous Glut4 deficient mice (Glut4 +/-), in contrast, display significant insulin resistance and diabetic histopathologies that resemble those of humans with type 2 diabetes [14]. The muscle-specific Glut4-knockout (MG4KO) mice develop hyperglycemia, glucose intolerance and insulin resistance at as early as 8 weeks of age [15]. Surprisingly, while the adipose tissue accounts for less than 10% of insulin-induced glucose uptake [16], the adipose-specific Glut4 knockout (AG4KO) mice develop metabolic defects comparable to those observed in the MG4KO mice [17]. More remarkably, specific overexpression of Glut4 in the adipose tissue of the MG4KO mice significantly reverses their insulin resistance and diabetes [18]. This suggests a crosstalk between different insulin-targeting organs [19, 20], highlighting the role of Glut4 in adipose tissue as a reliable sensor for whole body energy and nutrient status [21]. In concert, gain-of function of Glut4 in adipose tissue [22-24], or muscle [25] through specific transgenic overexpression leads to enhanced

insulin sensitivity and glucose tolerance. Taken together, these intriguing studies suggest Glut4 as a key determinant of whole body glucose homeostasis.

Glut4 cycling and regulation

Itinerary of Glut4 trafficking

In light of the importance of Glut4 in glucose homeostasis, numerous studies have attempted to dissect the molecular basis of its regulation by insulin. At least three potential mechanisms have been proposed, 1) the expression level through transcriptional and translational control; 2) the intrinsic transport activity via post-translational modification, or 3) the spatial localization by regulating translocation. The first evidence in line with the 'translocation hypothesis' came from two independent observations that insulin causes a large redistribution of the 'glucose transport activity' (now known as Glut4) from the intracellular fractions to the membrane fractions in rat adipocytes [26, 27]. Since this initial discovery, more and more evidences support the hypothesis that insulin increases glucose uptake primarily through the trafficking of Glut4 to the plasma membrane, rather than promoting the intrinsic activity of the transporter [28, 29].

In the basal state, Glut4 slowly but constitutively recycles between the cytoplasm and plasma membrane [30, 31], with a steady state distribution favoring the intracellular compartments—the intracellular tubule-vesicular elements, the early/sorting and recycling endosomes (RE), and the trans-golgi network (TGN) [9, 32]. Upon insulin stimulation, a fraction of these Glut4-containing vesicles shift to the plasma membrane,

these vesicles which will translocate rapidly in response to insulin are referred to as Glut4 storage vesicles (GSV). The GSV are thought to directly bud from both the RE [33] and the TGN [9], and rapidly move towards the cell periphery, along both actin cables and microtubules in response to insulin, with the assistance of the myosin [34-36] and kinesin [37, 38] molecular motors. These Glut4 vesicles then undergo tethering and fusion with the plasma membrane. These processes are separately mediated by two groups of evolutionally conserved proteins; the exocyst complex, which tethers the vesicles to the plasma membrane [36, 39-42], and the SNAREs (Soluble NSF activating protein receptor) and SNARE-associated proteins which drive the final step of fusion of the lipid bilayers [6, 32, 43]. This eventually leads to the extracellular exposure of Glut4 and efficient glucose transport into cells. Glut4 on the cell membrane is later endocytosed to the cytoplasm through clathrin-dependent [44, 45] and cholesterol-mediated [46, 47] mechanisms back into a sorting endosome compartment [29, 43] thus completing a cycle of trafficking (**Figure 1.2**).

Regulation of Glut4 trafficking by Insulin

Insulin causes a 10-fold increase of Glut4 level on the plasma membrane, from 5~10% of total Glut4 in basal state to ~50% after 20 minutes of insulin treatment [48]. This pronounced redistribution of Glut4 is the net result of the insulin effect on three main steps of the Glut4 cycle: endocytosis, intracellular distribution and exocytosis. In general, insulin accelerates the exocytosis rate of Glut4 by 10-20 fold, which

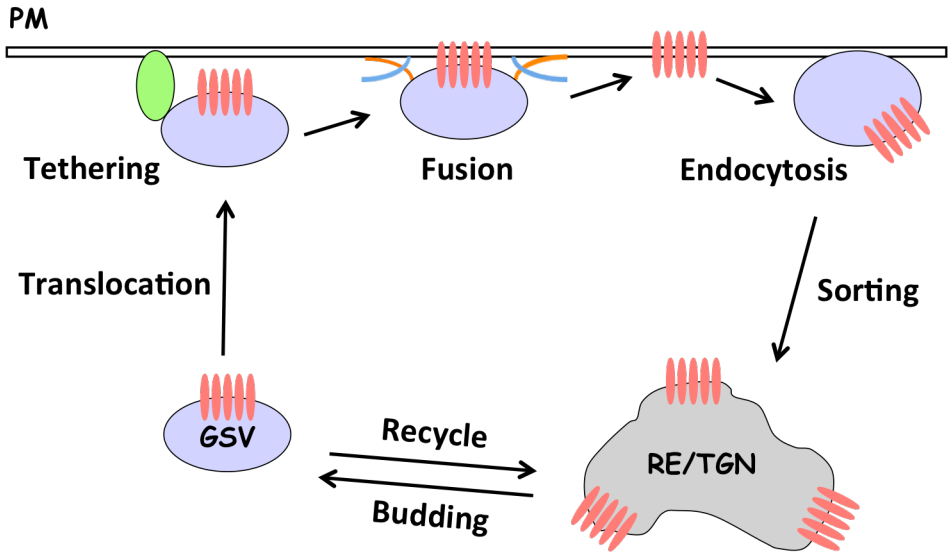


Figure 1.2 Itinerary of Glut4 trafficking. In the basal state, Glut4 is retained in the intracellular Glut4 Storage Vesicles (GSV), which undergo futile cycling with the recycling endosome (RE) and/or the trans-Golgi-network (TGN). Insulin stimulation breaks this futile cycle and mobilizes the GSV to translocate to the periphery of cell surface along the cytoskeletal tracks. The Glut4 vesicles then dock and fuse with the plasma membrane (PM), leading to robust extracellular exposure of Glut4 and consequentially efficient glucose uptake into cells. Glut4 on the plasma membrane is internalized back through clathrin- and caveolin- mediated endocytosis, and is re-sorted into RE/TGN to re-generate GSV. Courtesy of Dr. Dara Leto.

contributes most to the insulin-stimulated Glut4 translocation [6], while the significance and exact roles of insulin on Glut4 endocytosis and Glut4 intracellular redistribution are still being debated.

Glut4 endocytosis. Insulin stimulation decreases the rate of Glut4 endocytosis by ~2 fold in adipocytes but not muscle cells [47, 49]. While the mechanism of this decrease is still elusive, one study suggests that Glut4 is primarily internalized via a fast cholesterol-mediated process in unstimulated cells, but switches to a slow clathrin-dependent mechanism upon insulin stimulation [47]. This study, however, has been challenged by a recent report suggesting that the effect of insulin on Glut4 endocytosis no longer exists after being corrected for the concomitantly accelerated Glut4 exocytosis [50]. Therefore, the real effects of insulin on Glut4 endocytosis and the importance of the endocytic rate in regulating overall surface Glut4 level are still unclear and require further investigation.

Glut4 subcellular distribution. Two competing models have emerged regarding how insulin regulates the subcellular distribution of Glut4. The first model argues that all Glut4 is actively cycling in both basal and insulin-treated cells, and the effect of insulin is completely accounted for by changes of the rate of exocytosis and endocytosis [9, 51]. Several recent studies suggest a second model, the key feature of which is that after being internalized, a pool of Glut4 is sequestered in a specialized compartment referred as the Glut4 storage vesicles (GSV), which does not cycle between the cell surface and cell interior until summoned by the insulin signaling [52-54]. However, the exact identities and properties of GSV remain elusive, primarily due to the dynamic nature of these

membrane structures [33, 55] and a lack of GSV-specific markers. Nevertheless, a number of proteins have been reported to be integral components of the GSV, based on a combination of purification techniques including cell fractionation, compartment ablation, and immunoprecipitation with anti-Glut4 antibodies followed by immunoanalysis or mass spectrometry analysis. Among them, Glut4, IRAP [56-58], sortilin [59, 60], VAMP2 [61] and LRP1 [62] are identified as major GSV components.

Although the functions of these proteins in GSV are incompletely understood, VAMP2 has been proposed to be essential for fusion of GSV with the plasma membranes [63, 64], while sortilin, LRP1 and IRAP all positively regulate the formation of GSV [62, 65-67]. IRAP, the insulin-regulated amino peptidase, is implicated in the biogenesis and/or maintaining the integrity of the GSV [56-58]. This is consistent with observations from the IRAP-knockout mice, which show decreased Glut4 vesicles in heart, adipose tissue and muscle [68]. Interestingly, IRAP has been shown to interact with an Akt substrate AS160 [69, 70], a protein involved in regulating Glut4 trafficking, which will be discussed in detail below. Briefly, AS160 is phosphorylated in response to insulin by Akt, which may negatively regulate the function of AS160 [71]. It is also a negative regulator of the small GTPases Rabs8, 10 and 14 [72]. These Rabs play a regulatory role in vesicle trafficking, possibly through influencing GSV formation and/or intracellular retention [73-76]. Taken together, these pieces of evidence suggest insulin signaling may regulate Glut4 subcellular distribution by promoting the GSV formation through yet-to-be-defined mechanisms, which may involve AS160 and its target Rab proteins.

Despite the constitutive cycle between the plasma membrane and the cytosol, the majority of GSV localize in the cytosol in the basal state, indicating the existence of specialized machineries that retain these vesicles intracellularly in the absence of insulin. How this retention is achieved remains uncertain. Nonetheless, two mechanisms are proposed to be involved: anchoring and futile cycling. The anchoring mechanism suggests that proteins that may physically tether GSV to an unknown intercellular compartment. TUG (tether, containing a UBX domain, for Glut4) has been identified as one of these potential anchoring proteins, which has been shown to retain GSV within unstimulated cells possibly through an interaction with Glut4 [77]. While overexpression of TUG sequesters Glut4 away from trafficking, loss of TUG leads to enhanced Glut4 surface exposure [77, 78]. Interestingly, insulin stimulation seems to abrogate the interaction between TUG and Glut4 [77], suggesting that the first step of insulin action on the GSV could be releasing the anchor to release GSV into cycling. In addition to anchoring, GSV may also be sequestered in cells via a futile intracellular cycle of continuous budding from and fusion with the TGN and the recycling endosomes in the basal state, which could disfavor Glut4 cycling to the cell membrane [79]. The mechanisms underlying this phenomenon remain largely unclear, however the Rab31GTPase is proposed to contribute to the futile cycling of GSV [80]. Rab31 negatively regulates Glut4 translocation in adipocytes [80]; it also participates in TGN to endosome trafficking in other cell types [81]. Insulin could allow GSV to escape from this futile cycle by decreasing the Rab31 activity, through sequestration of Rab31 from its activator, the Rab31 GEF Gapex-5 [80].

Glut4 exocytosis. Exocytosis of GSV can be divided into three distinct steps: translocation towards the cell periphery, targeting to the membrane sites that contain the essential machinery for vesicle fusion, and finally fusion of the vesicle membrane with the plasma membrane. Numerous studies including the recent application of TIRF (Total Internal Reflection Fluoresence) microscopy [82-85] have shown that insulin possibly regulates every of these three processes, by recruiting different machineries that specifically facilitate each distinct step of the exocytotic journey of GSV.

Early studies with high-resolution imaging of Glut4 [86, 87] and drugs depolymerizing either actin [88, 89] or microtubules [90] support the involvement of the cytoskeleton network in Glut4 trafficking. Consistently, recent work suggests that several motor proteins including actin-based myosin [34-36] and microtubule-based kinesin [37, 38] are involved in GSV transport. Among them, the role of the unconventional myosin Myo1c in GSV mobility has been most extensively studied. Myo1c is recruited by insulin signaling to associate with actin filaments and actin-based GSV, and then drives the vesicles to sub-plasma-membrane region before their docking and fusion [34]. Myo1c dose not directly recognize GSV; instead, it is coupled to GSV via interaction with a GSV-localized small GTPase RalA upon insulin activation [36]. Another study indicates that insulin regulates Myo1c function via CaMKII-dependent phosphorylation, which is required for the motor activity of Myo1c in the process of GSV translocation [35].

In most, if not all, vesicular transport events, following the delivery by molecular motors on cytoskeleton tracks but preceding the final fusion, the vesicles are targeted through a

tethering step to the designated plasma membrane regions that contain the fusion machinery [91-93]. Several lines of evidence, including direct imaging with the TIRF microscopy, suggest that GSV are targeted to lipid rafts before the final insertion of Glut4 into the plasma membrane [42], and insulin increases the rate of this tethering/docking process to accelerate the external exposure of Glut4 [31, 83, 84]. A crucial component of the GSV tethering machinery is the exocyst, an evolutionarily conserved multimeric complex that regulates tethering events at the plasma membrane [94], including synaptogenesis [95], basolateral protein sorting [96] and Glut4 exocytosis [36, 39-42].

Insulin regulates exocyst-mediated GSV tethering mainly in three ways: recruitment of exocyst at the plasma membrane [41, 42], engagement of GSV with exocyst [36], and release of the exocyst to complete final fusion [39]. First, insulin increases exocyst assembly at the lipid rafts of plasma membrane by activating a small GTPase TC10 and promoting its interaction with the exocyst scaffolding subunit Exo70 [41], which in turn, brings other exocyst subunits in proximity of each other for assembly [42]. Also insulin activates a GSV-localized small GTPase RalA by phosphorylating and inhibiting the Akt substrate RGC1/2, a complex that specifically inactivates RalA [97]. Upon activation, RalA interacts with the exocyst subunits Sec5 and Exo84 [36, 98, 99], engaging GSV with the exocyst. Last, for final fusion to take place, GSV need to break with the large tethering exocyst complex [92]. This disengagement is achieved through insulin-stimulated, PKC-dependent phosphorylation of the RalA-binding exocyst subunit Sec5. This phosphorylation dislodges RalA and GSV from the exocyst, and is regarded as a gatekeeping step in controlling GSV fusion [39] (**Figure 1.3**).

In addition to RGC1/2, another Akt substrate AS160 has also been implicated in regulating the tethering/docking of GSV to the plasma membrane. This function of AS160 has largely emerged from TIRF microscopy experiments performed using a non-phosphorylatable mutant of AS160, AS160 4A, which is regarded as a constitutively active form of AS160 [71]. Adipocytes overexpressing AS160 4A show proportionally decreased tethering and fusion of GSV to the plasma membrane, indicating an inhibitory role of AS160 in the docking of GSV but not fusion after docking [82, 84, 100]. Insulin probably reverses this negative effect of AS160 by Akt-catalyzed phosphorylation. However, unlike RGC1/2, the intracellular substrates of AS160 have not been characterized yet, therefore the exact function of AS160 in regulating GSV translocation also remains to be elucidated.

The final step of the GSV exocytosis is the fusion between the vesicle membrane and plasma membrane, a process catalyzed, like all other fusion events, by the SNARE complex [101, 102]. According to their different roles, the SNARE complex can be categorized into two classes: the v-SNAREs that localize on the donor/vesicle membrane, and the t-SNARE that dwell on the acceptor/target membrane [103]. Membrane fusion

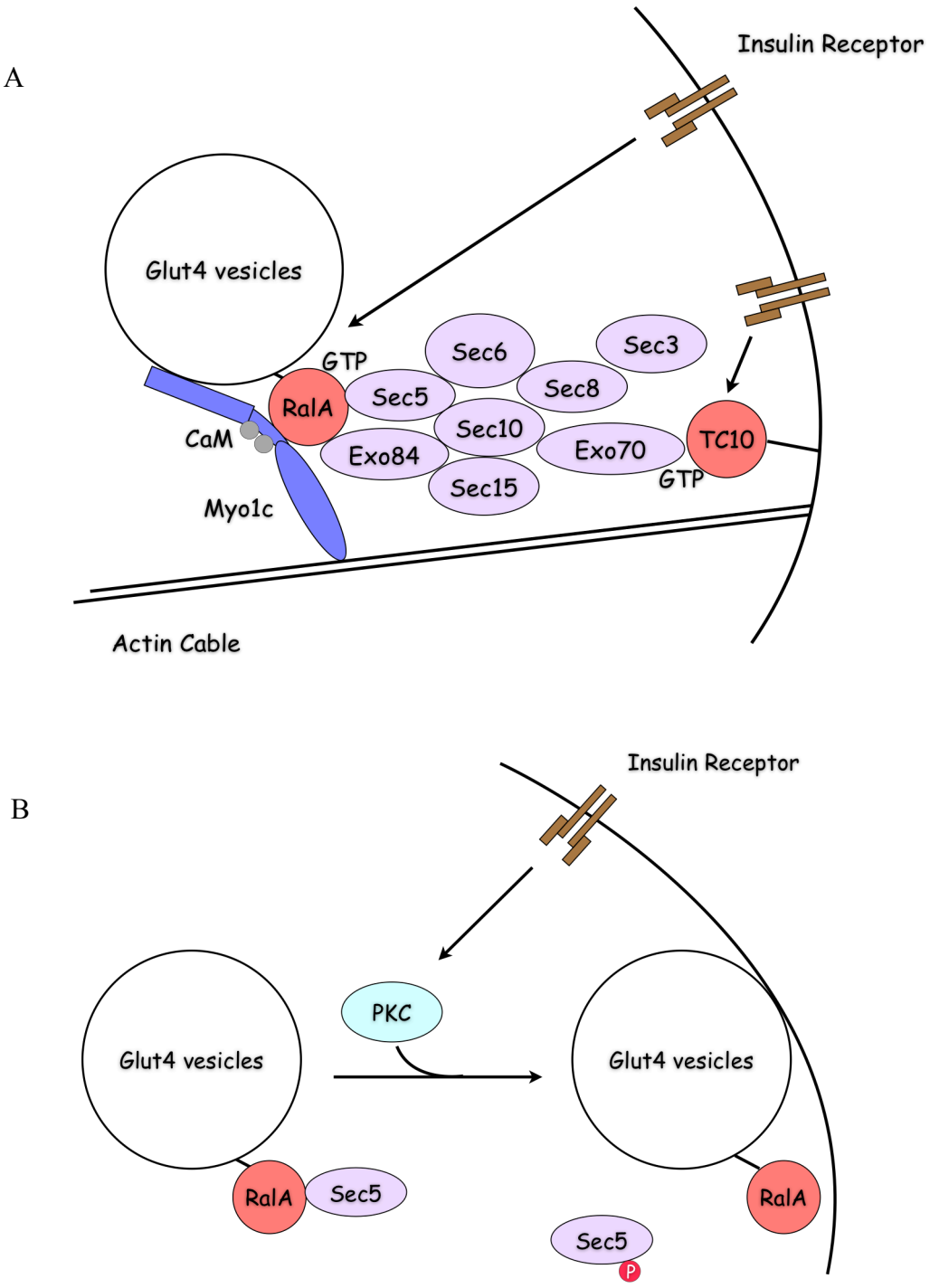


Figure 1.3 The exocyst complex in Glut4 vesicle exocytosis. (A) Insulin-stimulated exocyst assembly requires activation of RalA on the Glut4 vesicles and TC10 on the plasma membrane. RalA moves the Glut4 vesicles along actin tracks via motor protein Myo1c and calcium/calmodulin to sites for docking that are marked by active TC10 and TC10 associated exocyst components on the plasma membrane. (B) Membrane fusion occurs after exocyst complex is disengaged from Glut4 vesicle through PKC-catalyzed phosphorylation on Sec5, which disrupts the interaction between Sec5 and RalA. Summarized from [36, 39, 41, 42].

occurs when the v-SNARE and the cognate t-SNARE forms a ternary complex and generates driving force to overcome the energetic barrier of fusion [104, 105]. SM (Sec1/Muc18-like) proteins play complementary role to SNAREs and direct their fusogenic action [106]. To date, the SNARE and SNARE-associated proteins identified in regulating GSV fusion are the GSV-localized v-SNARE VAMP2 [107], the plasma membrane-localized t-SNARE Syntaxin-4 and its accessory protein SNAP-23 [64, 107-109]. While these proteins are considered as the minimal machinery requirement for membrane fusion, their functions are also regulated by regulatory proteins such as Synip and Munc18c [29]. Synip was identified as a Syntaxin-4 binding protein in a yeast two-hybrid screen [110]. Insulin stimulation decreases the interaction between Synip and the t-SNARE Syntaxin-4, which may free Syntaxin-4 to bind with the v-SNARE VAMP2 on the incoming GSV [110]. It was proposed that insulin regulates Synip through an Akt2-dependent phosphorylation [111], while the significance of this phosphorylation remains controversial [112, 113]. The SM protein Munc18c is one isoform of the Munc18 family that binds to Syntaxin-4 on the plasma membrane in adipocytes [114]. After the characterization of this interaction, extensive studies have been performed to investigate the role of Munc18c in GSV exocytosis, which revealed contradictory results indicating Munc18c as both an inhibitory [115-118] and a necessary [119-121] component in regulating GSV fusion. In addition, how insulin regulates Munc18c function is also unclear. Some studies have reported that insulin changes the binding pattern between Munc18c and Syntaxin-4, permitting SNARE complex formation and vesicle fusion [122, 123]. Nevertheless, other observations suggest that insulin stimulation leads to Munc18c phosphorylation and consequently dissociation from Syntaxin-4 [124]. In summary,

although Munc18c is likely may play a role in GSV fusion with the plasma membrane, the exact mechanism involved in this process is complicated and not completely understood.

II. Insulin Signaling that regulates Glut4 trafficking

Despite decades of effort, our understanding of the insulin signaling events governing Glut4 vesicle translocation remains incomplete. Insulin action is initiated when the hormone binds to its cell surface receptor, which belongs to a subfamily of tyrosine kinase receptors that consist of two α and two β subunits [125]. Upon binding of insulin to the extracellular α subunits, the transmembrane β subunits transphosphorylate each other on three specific tyrosine residues in an area called activation loop. This increases the tyrosine kinase activity of the β subunits, which further autophosphorylate other tyrosine residues in the juxtamembrane regions and the cytosolic tails, and result in full activation of the receptor tyrosine kinase [126-128]. Once activated, the insulin receptor recruits and phosphorylates numerous substrate proteins on specific tyrosine residues. To date, the identified substrates include the IRS1-6 (insulin receptor substrates family), Gab-1, SHC, Cbl, Grb, APS, and SIRP family members [127, 128]. The phosphorylated tyrosine residues within these substrate proteins then serve as 'docking sites' for downstream effector and adaptor proteins containing the SH2 (Src homology 2) domains to interact with, creating a platform for the signals from the insulin receptor to diverge and integrate in with multiple signaling cascades [127-130].

PI 3-kinase signaling pathway

IR-IRS-PI3K-PIP_3-PDK1-Akt axis

The IRS family of adaptor proteins are the best characterized insulin receptor substrates [130, 131]. Upon tyrosine phosphorylation by the insulin receptor, IRS proteins recruit SH2-domain containing proteins to the plasma membrane, including the class IA PI 3-kinases (phosphatidylinositol-3-kinases). The interaction between IRS and the regulatory subunit (p85) of the PI 3-kinase recruits the catalytic subunit (p110) and results in the activation of the kinase [5, 132]. In fat and muscle cells, IRS-1 is considered as the major IRS family member involved in the PI 3-kinase activation [133]. Activated PI 3-kinase generates the lipid product PI(3,4,5)P3 (phosphatidylinositol 3,4,5-trisphosphate) by phosphorylating PI(4,5)P2 (phosphatidylinositol 4,5-bisphosphate) at the 3 position [134]. PI(3,4,5)P3 acts as a docking site for several PH (pleckstrin homology) domain-containing proteins to localize to the plasma membrane, which is vital to the activation of these proteins and the initiation of downstream cellular events [134]. One of the proteins recruited by PI(3,4,5)P3 is the AGC (protein kinase A/protein kinase G/protein kinase C-family) kinase family member PDK1, which in turn phosphorylates and activates additional kinases including Akt1-3, some PKCs and SGK (serum and glucocorticoid-inducible kinases) [135]. Akt is also an AGC kinase that contains a PH domain at its N-

terminus, which binds to PI(3,4,5)P3, and presents Akt to PDK1, enabling phosphorylation at threonine 308 in the Akt kinase domain by PDK1 [136, 137]. Full activation of Akt also requires phosphorylation of serine 473 by mTORC2 (mammalian Target of Rapamycin complex 2) [138-140], which consists of mTOR, a member of the PI3K-related family of kinases and several adaptor proteins [141, 142]. Over the past years, new components of the mTORC2 have been identified and the list is still expanding. Among the most studied components, Rictor (rapamycin-insensitive companion of mTOR) serves as a scaffold protein that facilitates the assembly of the complex and the interaction between mTORC2 and substrate proteins [139, 143, 144]; mLST8 (mammalian lethal with SEC13 protein 8) stabilize the interaction between Rictor and mTOR [145]; and mSIN1 has been proposed to facilitate mTORC2 translocation to the plasma membrane via the C-terminal PH domain, and eventually phosphorylation and activation of Akt [142, 146]. Once activated, Akt phosphorylates multiple substrate proteins at a consensus motif RXRXXS/T [147-149]. Upon phosphorylation, Akt substrates are activated or inactivated, exerting regulation over a variety of cellular processes, including Glut4 trafficking.

The pivotal role of IRS-PI 3-kinase-Akt signaling in insulin stimulated-glucose transport has been supported by numerous studies over the past decades. While knockdown of IRS-1 in rat adipocytes causes increase in the concentration of insulin required to achieve half-maximal stimulation of Glut4 translocation, overexpression of human IRS-1 in these cells largely increases the basal Glut4 surface exposure and left shifts the insulin-dose response curve [150], suggesting a functionally significant role of IRS-1 in insulin-

stimulated Glut4 translocation. In addition, inhibition of the enzyme activity of PI 3-kinase with pharmacological inhibitors such as wortmannin or LY294002, or introducing into cells the dominant-negative form of PI 3-kinase completely blocks insulin-stimulated glucose uptake and Glut4 translocation, whereas overexpression the active form of PI 3-kinase partially mimics the insulin action [151-154]. Furthermore, genetically knockout the catalytic subunits of PI 3-Kinases in mice results in glucose intolerance and insulin resistance, while ablation of the regulatory subunit of PI 3-kinase in mice leads to enhanced glucose uptake and improved insulin sensitivity, potentially due to removal of inhibition on the kinase activity [155-157]. Together, these studies have established the essential role of PI 3-kinase in glucose uptake and energy homeostasis. Finally, activation of Akt by PI 3-Kinase has long been considered as a critical step in Glut4 translocation, based on evidence from cell biology studies and genetically engineered mice models. For instance, overexpression of a membrane-bound thus constitutively active form of Akt in 3T3-L1 adipocytes increases glucose transport and Glut4 translocation [158], whereas overexpression of a dominant-negative mutant of Akt or depletion of the protein via siRNA blocks insulin-stimulated Glut4 translocation [159-161]. There are three highly homologous mammalian isoforms of Akt (Akt1-3), which despite their similar primary sequences, are not functionally redundant [162]. Among the three isoforms, Akt2 appears to be the major player in regulating glucose transport, as knockdown of Akt2 in 3T3-L1 adipocytes significantly blocks insulin-stimulated glucose uptake, whereas knockdown of Akt1 shows little effect [161]. Consistent with this, mice lacking Akt1 are smaller but display normal glucose tolerance, while Akt2 knockout mice become insulin resistant and overtly diabetic with aging [163, 164]. Moreover, Akt3-null mice exhibit normal glucose

metabolism, and the double knockout of Akt2 and Akt3 mice have a similar metabolic phenotype to Akt2 knockout mice, suggesting Akt3 may not play a significant role in glucose homeostasis [165, 166].

Among numerous identified substrates of Akt kinases, only a few have been implicated in regulating insulin-stimulated Glut4 trafficking [167]. With an antibody recognizing the phosphorylated consensus motif (RXRXXpS/pT) in Akt substrates, Lienhard and coworkers identified two novel Akt substrates in 3T3-L1 adipocytes, AS160 [168] and AS250 (also known as RGC2) [169]. Interestingly, in addition to Akt substrate motifs which are phosphorylated in response to insulin, both proteins also contain GTPase activating protein (GAP) domains, which may function as negative regulators for certain small GTPases [168, 169]. Studies regarding the role of these two Akt substrates will be discussed in details in the following paragraphs.

Akt substrate AS160

AS160 was originally known as TBC1D4, and together with its muscle isoform TBC1D1, belongs to the TBC1 (tre-2/USP6, BUB2, cdc16) domain family. The TBC1 domain is a putative Rab GAP domain that may possess catalytic activity to certain Rab GTPases [170]. AS160 also contains at its N-terminus two PTB (phosphotyrosine-binding) domains with unknown function, and one CBD (calmodulin-binding domain) in between the second PTB domain and the TBC1/GAP domain [171]. The CBD domain may be important in regulation contraction- but not insulin-stimulated glucose transport in

muscle cell [172], while studies with insulin in 3T3-L1 adipocytes failed to reveal any functional role of this domain [173]. Motif scan and mass spectrometry phosphopeptide analysis of the AS160 protein has identified six phosphorylation sites matching the Akt consensus motif and showed that levels of phosphorylation at five of these sites was increased in response to insulin, including Ser318, Ser570, Ser588, Thr642, and Thr751 (in human AS160) [71]. Additional studies have reported that AS160 can be phosphorylated by other AGC kinases including SGK1, RSK1 (p90 ribosomal S6 Kinase 1), and AMPK (adenosine monophosphate-activated protein kinase), in response to non-insulin stimuli such as exercise, growth factors and the AMPK activator AICAR [174-177]. Nonetheless, the functional significance of the phosphorylation of AS160 by these stimuli remains poorly understood.

The role of AS160 in regulating Glut4 trafficking has been largely revealed by studies with the AS160 4A mutant, in which four of the Akt phosphorylation sites (Ser318, Ser588, Thr642, Ser751) have been mutated to the non-phosphorylable alanine [71]. While overexpression of the wild-type AS160 shows little effect, overexpression of AS160 4A reduces insulin-stimulated Glut4 translocation by as much as 80% [71]. Moreover, simultaneously disrupting the putative GAP domain in AS160 4A by substituting a key arginine 973 residue with lysine (AS160 4A/RK mutant) completely reverses the inhibitory effect on Glut4 translocation [71]. These data, taken together, indicate that 1) AS160 plays a negative role in Glut4 trafficking, potentially through its GAP catalytic activity towards certain Rab GTPase(s); 2) Akt phosphorylation negatively regulates the GAP activity of AS160, releasing the downstream Rab GTPase(s) from

inhibition. Consistent with this model, siRNA-mediated depletion of AS160 in 3T3-L1 adipocytes increases basal Glut4 translocation and glucose uptake, an effect that can be reversed by re-introducing the wild-type AS160, but not the GAP-deficient AS160 RK mutant, suggesting that AS160 retains Glut4 in the intracellular compartments in a GAP activity dependent manner [178, 179]. It is noteworthy that among the five Akt phosphorylation sites, Thr642 is considered to be the most functionally important site, as a single mutation of Thr642 to alanine decreases insulin-stimulated Glut4 translocation by 60% [71]. Intriguingly, mice with the AS160 Thr649 (equivalent Thr642 in human) to alanine knock-in mutation display impaired insulin sensitivity and decreased muscle glucose uptake *in vivo*, indicating insulin-induced phosphorylation on AS160 plays an important role in regulating whole-body glucose homeostasis, at least partially through regulating Glut4 trafficking [180].

To test whether the predicted GAP domain in AS160 has any physiological function, Lienhard and coworkers sought the target Rab GTPases through an *in vitro* GTP hydrolysis assay with the isolated AS160 GAP domain [72]. This is not an ideal situation, as the full-length AS160 may be expected to behave differently from the GAP domain alone. Nonetheless, among the 18 Rab GTPases screened, they demonstrated that the recombinant AS160 GAP domain selectively accelerates the GTP hydrolysis of Rab2, Rab8A, Rab10 and Rab14 but not the others [72]. Among these candidate substrates, Rab10 has emerged as the Rab most likely to be involved in Glut4 trafficking in 3T3-L1 adipocytes. Overexpression of the constitutively active Rab10 mutant causes an approximately 2-fold increase in the amount of Glut4 at the cell surface in basal state [74].

In addition, knockdown Rab10 but not Rab8A or Rab14 in 3T3-L1 cells significantly decreases insulin-induced Glut4 translocation [74, 76]. In contrast, in L6 muscle cell lines, Rab8A and Rab14 but not Rab10 appear to be required for Glut4 translocation [181].

Evidence that Rab8A, Rab10 and Rab14 may be potential functional targets of AS160 comes from several studies in which the effect of AS160 on Glut4 trafficking was modified by introduction or depletion of these Rab GTPases. For instance, knockdown of AS160 results in 3T3-L1 adipocytes with increased basal Glut4 translocation, while simultaneous ablation of Rab10 partially blocks the increased Glut4 translocation [74]. In another study, overexpression of AS160 4A reduced insulin-stimulated Glut4 translocation in L6 muscle cells, but co-overexpression of Rab8A or Rab14 but not Rab10 with AS160 4A could restore insulin-stimulated Glut4 translocation [181]. From these data, it is tempting to conclude that Rab10 and Rab8A/Rab14 may be the targets of AS160 in regulating insulin-stimulated Glut4 trafficking in adipocytes and muscle cells respectively. Nevertheless, the biggest caveat of these rescue experiments is that they don't provide the evidence that AS160 functionally regulates these Rab GTPases. It is possible that AS160 and Rab10 may be in two parallel pathways that each contributes independently to Glut4 translocation. Therefore, further studies are required to demonstrate the direct regulation of AS160 on Rab10. In chapter 2 of this dissertation, I will establish Rab10 as a direct *in vivo* target of the AS160 GAP activity. It has also long been assumed that the Akt phosphorylation results in decreased GAP activity of AS160. However, there have been no reports of this hypothesis being directly tested, or evidence

linking the phosphorylation to any potential Rab targets. I will also demonstrate that phosphorylation of AS160 by Akt reduces its GAP activity towards Rab10.

Akt substrate RGC1/2

Another Akt substrate AS250 was originally identified as a protein with an apparent molecular weight of 250 kDa on a SDS-PAGE gel, which contains a putative GAP domain homologous to the Rheb GAP TSC2 (Tuberous Sclerosis Complex 2) [169]. TSC2, in complex with TSC1, is an extensively studied Akt substrate that has been implicated in regulating cell growth, proliferation and energy status through the small GTPase Rheb and its effector the mTORC1 (mammalian Target of Rapamycin complex 1) [182, 183]. Upon phosphorylation by Akt, TSC2's GAP activity towards Rheb is inhibited, leading to increased activity of Rheb and consequentially the activation of mTORC1 [184-187]. Interestingly, AS250 was also found to exist in stable complex with another protein KIAA1219, in a manner reminiscent of the TSC1/TSC2 complex [169]. These two proteins were thus renamed as RGC1 and RGC2 to reflect their function and homology with TSC1/2. KIAA1219/RGC1, like TSC1, with no characterized catalytic domains, and represents the regulatory subunit of the complex; AS250/RGC2 contains the GAP domain and represents the catalytic subunit of the complex [97].

Upon the isolation of the RGC1/2 complex, Leinhard and coworkers also searched for GAP activity of RGC2 toward several potential small GTPases substrates such as Rheb, Rap1A, RalA, and Ras, but failed to detect any GAP activity [169]. This was probably

because the isolated complex in their study was not in a native, active conformation; as in an effort to search for GAP proteins for the RalA GTPase, two later studies simultaneously and independently identified the RGC1/2 complex as the long missing GAP for RalA [97, 188]. As discussed before, RalA is rapidly activated by insulin in a PI 3-kinase dependent manner in adipocytes and plays a critical role in regulating insulin-stimulated Glut4 vesicle trafficking through its association with the exocyst complex and the myosin motor Myo1c [36]. These data together render the RGC1/2 complex a missing link between the PI 3-kinase-Akt signaling and the activation of RalA and the final mobilization of Glut4 vesicles. Indeed, siRNA depletion of the RGC proteins causes increased RalA activity and glucose uptake and increased plasma membrane Glut4 level in adipocytes [96, 259], indicating the RGC1/2 complex plays a negative role in Glut4 trafficking by keeping RalA in the inactive state. In response to insulin, Akt2 directly phosphorylates RGC proteins. Instead of directly hampering the catalytic activity of the RGC2, this phosphorylation is thought to cause a conformational modification of RGC1/2 that decreases the interaction between the complex and RalA, leading to the activation of RalA and eventually Glut4 vesicle translocation [96].

APS signaling pathway

Growing evidence suggests that a PI 3-kinases-independent signaling pathway, restricted to lipid rafts, also regulates Glut4 vesicle exocytosis [189-191]. Lipid rafts are microdomains of the plasma membrane that are enriched in cholesterol, sphigolipids, glycolipids, lipid-modified signaling proteins, and multiple signaling proteins including

the insulin receptor [192, 193]. Interestingly, interruption of the formation of these special lipid rafts in mature adipocytes causes disrupted insulin receptor localization, insulin signaling and glucose uptake [193].

Subsequent studies have characterized the lipid raft-localized PI 3-kinase-independent insulin signaling pathway, which is initiated when the adaptor protein APS (adaptor protein containing PH and SH2 domains) is recruited to the activated insulin receptor [194]. APS exists as homodimer, and in contrast the most of the insulin receptor substrates which bind to the juxta-membrane regions of the receptor [127, 128], APS binds to the activation loop of the receptor through the interaction between the phosphorylated tyrosine residues on the receptor and the SH2 domain of APS [195, 196]. Upon binding, APS is phosphorylated on a tyrosine residue at its C-terminus, providing a docking site for the N-terminal variant SH2 domain of the adaptor protein Cbl [197, 198]. Cbl is in constant association with another adaptor protein CAP (Cbl Associated Protein), which interacts with the integral lipid raft protein Flotillin and thus anchors the Cbl-CAP complex in lipid rafts [199-201]. Cbl also contains numerous tyrosine residues that could serve as docking sites for other SH2-containing molecules upon phosphorylation. Indeed, upon binding to APS, Cbl undergoes phosphorylation by the insulin receptor on three tyrosine residues, and recruits the adapter protein Crk to lipid rafts through the SH2 domain of the latter protein [197-199]. Crk associates with C3G, a protein with several proline-rich sequences binding to SH3 domains within Crk, and a GEF (guanine nucleotide exchange factor) domain at the C-terminus [202, 203]. The GEF domain of Crk can catalyze the activation of the small GTPase TC10, which resides in lipid rafts

microdomains of 3T3-L1 adipocytes [204, 205]. Therefore, upon insulin stimulation, C3G is recruited to the lipid rafts in close proximity to its target small GTPase TC10, through the APS-CAP/Cbl-Crk adaptor proteins, which leads to a rapid activation of TC10 by insulin [204]. Active TC10 serves as a critical regulator of Glut4 exocytosis through interactions with several downstream effector proteins including CIP4, Par6 and Exo70 [28] (**Figure 1.4**).

The adaptor protein CIP4 belongs to the F-BAR protein family that contains multiple domains, including an N terminal F-BAR domain, an HR1 coiled-coil domain, and a C-terminal SH3 domain [206]. CIP4 acts as an effector of both Cdc42 and TC10 due to its preferential interaction with the GTP-loaded form of Cdc42 [207] and TC10 [208]. Upon insulin stimulation, CIP4 is recruited from the cytosol to the plasma membrane through a direction interaction with the activated TC10, while deletion of the TC10-binding domain of CIP4 blocks its movement towards the plasma membrane as well as Glut4 translocation [208]. Through its SH3 domain, CIP4 is able to associate with the proline-rich sequence in the middle of Gapex5, which also contains a RasGAP domain at its N-terminus and a VPS9/RabGEF domain at its C-terminus [80]. As discussed before, the VPS9 domain facilitates the GTP-loading of the Rab5 family member Rab31, which retains Glut4 within an intracellular futile cycle in the basal state. Insulin-stimulated activation of TC10 is able to recruit Gapex-5 to the plasma membrane along with CIP4.

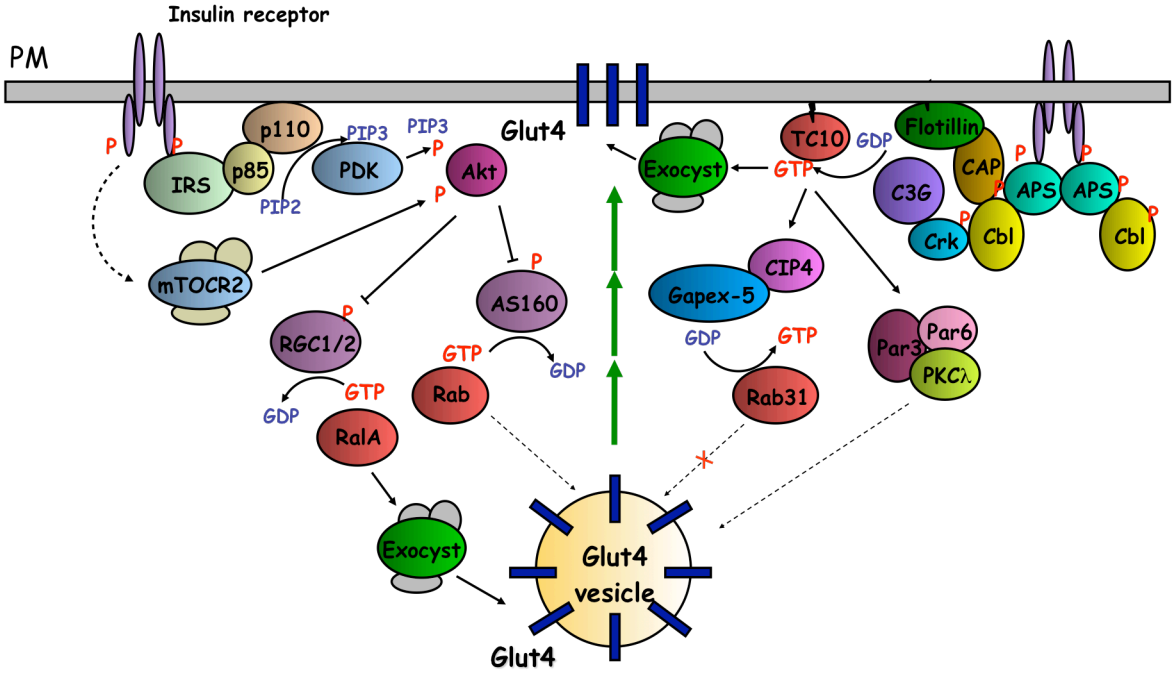


Figure 1.4 Insulin signaling pathways that regulate Glut4 trafficking. Upon insulin stimulation, multiple signaling cascades are initiated through the hormone receptor and the receptor substrates. The PI 3-kinase-dependent signaling pathway eventually results in activation of protein kinase Akt. Akt at least regulates two substrates that are implicated in Glut4 trafficking. One substrate is the RGC1/2 GAP complex that inactivates its substrate small GTPase RalA. Akt phosphorylation on RGC proteins inhibits the GAP activity of the complex and thus causes RalA activation. RalA regulates Glut4 trafficking through motor protein Myo1c and exocyst complex components Exo84 and Sec5. Another Akt substrate AS160 also contains GAP activity towards some Rab GTPases, which may be required for Glut4 trafficking. The PI 3-kinase-independent pathway eventually leads to the activation of small GTPase TC10, which regulates Glut4 trafficking by mobilizing downstream effectors including the CIP4/Gapex-5 complex, the exocyst complex subunit Exo70 and the Par3/Par6/aPKC complex. Modified from Chang *et al.*, Mol Med, 2004. **10**(7-12): p. 65-71.

This translocation on one hand, decreases Rab31 activity by sequestering it from its activator Gapex-5, and increases Glut4 exocytosis by permitting it to escape from the futile cycle [80]. On the other hand, once recruited to the plasma membrane, Gapex-5 activates another Rab5 family member Rab5, and thus increasing PI3P formation at the plasma membrane, a process that is potentially important for insulin-stimulated Glut4 trafficking [118, 209-211].

Another identified effector of TC10 is Par6 from the Par3/6 adaptor complex that forms a complex with atypical PKCs [212, 213]. Atypical PKCs have been shown to regulate insulin-stimulated glucose uptake [214, 215]. One study also reveals the involvement of Par3 and Par6 in glucose transport [213]. The same study also shows that active TC10 is able to recruit aPKC to the plasma membrane through their common association with Par6 and Par3 [213]. However, it is still unclear how exactly does the Par3/6/aPKC complex function to regulate Glut4 trafficking. One possibility is that the Par3/6/aPKC may regulate the Glut4 vesicles motility through the microtubule motor KIF3/kinesin II [216, 217].

Activated TC10 also interacts with Exo70, a component of the exocyst complex [41, 218]. Upon activation by insulin, TC10 recruits Exo70 to the plasma membrane, where they together recruit the exocyst subunits Sec6 and Sec8 (and perhaps other exocyst proteins) [41, 42]. Therefore, activation of TC10 is thought to be critical for the onsite exocyst assembly, which directs the tethering and eventually fusion of Glut4 vesicles to the plasma membrane. In agreement with this, a block in Exo70 interaction with TC10 does

not prevent Glut4 vesicles from translocating to the cell periphery, but instead blocks the final steps of Glut4 exocytosis—tethering and fusion [41, 42]. Interestingly, GTP hydrolysis of TC10 is also required for the final fusion to take place [219], indicating that exocyst disassembly via TC10 inactivation is also a crucial step of Glut4 transport.

Ras signaling pathway

Another substrate recruited by the tyrosine-phosphorylated IRS-1 is the SH2 domain-containing adaptor protein Grb2, which forms a stable complex with a Ras GEF protein SOS [220]. This Grb2-mediated translocation of SOS (Son of Sevenless) to the plasma membrane brings the GEF to where Ras resides, leading to the activation of the small GTPase [220, 221]. Activated Ras stimulates multiple downstream signaling cascades, among which the best studied pathway is through activation of the Raf kinase, mitogen-activated protein kinase kinase (MAPKK) and MAPK axis [222], which is a key signaling pathway critical for many facets of Ras biology [223]. The role of this pathway in metabolic actions of insulin is controversial [224-227]. Studies with inhibitors targeting this signaling pathway indicate that MEK/MAPK has little, if any, impact on insulin-stimulated Glut4 translocation [226, 228].

The RalGDS family members are another class of Ras effectors, which have gained special attention because of their involvement in multiple Ras regulated cellular processes [229]. The RalGDS proteins all contain a Ras-binding domain that interacts with GTP-bound Ras, and a CDC25 homology domain that possesses catalytic activity

for activation of the Ral small GTPases [229]. As discussed before, RalA has been reported to play a regulatory role in insulin-stimulated Glut4 translocation [36, 39, 97]. It is therefore possible that Ras may also be implicated in Glut4 transport through regulating RalGDS-RalA. Further study in this regard may reveal interesting roles of Ras in insulin action and Glut4 trafficking independent of MAPK signaling.

III. Roles and Regulations of Small GTPases in Glut4 Trafficking

Two most fundamental questions in the study of insulin regulated Glut4 trafficking are: 1) How is the specificity of the vesicle transport achieved? In other words, what factors guarantee that the final fusion event happens only at the appropriate time and in the right place? 2) How is the hormonal signal of insulin ultimately transduced to the physical movement of the vesicles [167]? Although not completely understood, emerging evidences suggest that small GTPases may provide some answers to both questions [127, 230].

The small GTPase superfamily exist in eukaryotes from protozoan to human and consists of over 160 low molecular weight (20-40 kDa) proteins [231]. The superfamily is named by its founding member Ras proteins, which are frequently mutated in some human carcinomas, and is divided into five subfamilies based on the structural and functional similarities of the member small GTPases: the Ras, Rho, Rab, Sar1/Arf, and Ran families [231]. The subfamilies take part in a vast number of different cellular processes. The Ras

subfamily members regulate gene expression, cell proliferation and differentiation. The Rho subfamily members are in charge of cytoskeletal reorganization, cell morphology and migration. The Rab subfamily members control the multiple steps of intracellular vesicle trafficking, including translocation, targeting and fusion. The Sar1/Arf subfamily members are involved in vesicle budding and formation. Finally, the Ran members function mainly in nucleocytoplasmic transport [231].

Structural basis of small GTPases function

Despite their distinctive roles in regulating cellular events, all the small GTPases possess a consensus sequence responsible for GDP/GTP binding and GTP hydrolysis, which is the base of their physiological function as 'molecular switches'. Crystallographic and NMR analysis of some small GTPases from the Ras, Rho, Rab, Arf and Ran subfamilies have revealed a common topology of the GDP/GTP binding domain (G-domain), which also constitutes the catalytic core of these enzymes [232]. This G-domain contains of 5 peptide loops (G1-5) that are responsible for different functions. The G1 loop (also called the P-loop) interacts with the α- and β- phosphate groups of the GTP. The G4 and G5 loops are responsible to recognize the guanosine base. The G2 and G3 loops, along with their ajacent amino acids constitue two 'switch' regions that undergo conformational change upon GTP binding and hydrolysis [233]. The G2/switch I and G3/switch II are bound to the γ-phosphate of GTP via the concerved Thr and Gly residues in each switch. This interaction with GTP stablize the switch regions in a conformation that enables the small GTPase to bind to its specific downstream effectors, mainly through the switch I

region. However, release of the γ-phosphate after GTP hydrolysis breaks the hydrogen bonds between the switches and the bound neucleotid, which allows the switch regions to relax into a different conformation that loses interaction with the effectors [233-235] (**Figure 1.5A**). An effector of a small GTPase is defined as a protein or protein complex that interacts with the small GTPase directly and in a GTP-dependent manner and is required for the downstream function determined by that small GTPase [236].

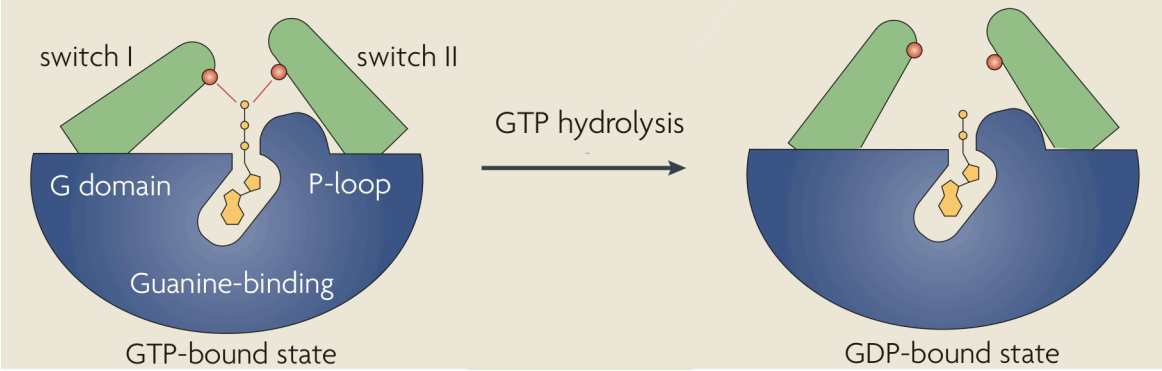
In summary, the most important biochemical feature of small GTPases is their ability to oscillate between two conformational states—the GTP bound state and GDP bound state. When binding to GTP, small GTPases are able to recruit certain effectors and trigger respective downstream events, which is therefore regarded as the 'active' state of the small GTPases; after GTP hydrolysis by their intrinsic GTPase activity, small GTPases disassociate with effectors and terminate the previously triggered events, which is accordingly described as the 'inactive' state of the small GTPases.

If most of the small GTPases behave in the very similar way, what make them distinctive from each other? Intriguingly, sequence alignment of small GTPases within and across subfamilies reveal that two highly flexible regions that may contribute most to their diversity. The first flexible region is the 'switches' within the otherwise conserved G-domain. A structural comparison of G-domains shows that the G1 phosphate recognition loop and the G4/G5 nucleotide-binding region are unsurprisingly highly conserved; while the G2/switch I region responsible for downstream effector interaction, and the G3/switch II region for γ-phosphate interaction are much more diverse. The conformational

differences in the switch regions thereby directly affect the ability of different small GTPases to bind to their distinctive effectors and the intrinsic knetics of GTP hydrolysis [233].

The second highly flexible region is carboxy-terminal region that may undergo post-translational lipid modifications, which serve as targeting signal to localize each small GTPase to a specific membrane. For instance, the majority of Ras subfamily proteins possess a C-termainal CAAX (C=Cys, A=aliphatic, X=any amino acid) tetrapeptide, which is recognized by lipid transferases and undergoes farnesylization or geranylgeranylization on the cysteine residue. This lipid modified motif, coupled with redisues immediately upstream (e.g. cysteine residues with palmitoylation), consists of the membrane-targeting sequences that mediate interactions with membrane phospholidids, localizing the Ras proteins at the cytoplasmic face of the plasma membrane [237, 238]. In contrast, most of the Rab subfamily proteins have a distinct set of cysteine-containing C-terminal motifs (mostly CC and CXC) of which Cys residues are geranylgeranlyated and able to localize the Rab proteins to distinct membranes of the intracelluar vesicle trafficking system [231, 238, 239] (**Figure 1.5B**). Therefore, the C-terminal regions and lipid modifications are considered critical for the distinctive biological activities of the small GTPases, since their regulatory functions are restriced to the membrane compartements where they are localized.

A



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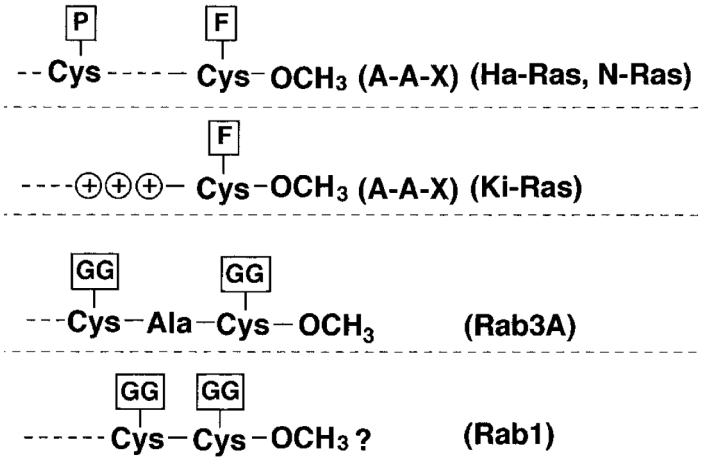

Figure 1.5 Biochemical features of small GTPases. (A) Conformation of the G domain of small GTPases. Guanosine base binds in the pocket formed by the G4 and G5 loops. The G1 loop (also called the P-loop) interacts with the α- and β- phosphate of the GTP (and GDP). The G2 and G3 loops form the switch I and switch II regions, which bind to the γ-phosphate of GTP and provide an interface for downstream effectors to bind. Release of the γ-phosphate after GTP hydrolysis causes the switch regions to assume a different conformation, thus losing interaction with the effectors. Adapted from Weirich *et al.*, Nat Rev Mol Cell Biol, 2008. **9**(6): p. 478-89. (B) Distinct C-terminal sequences and lipid modifications of different small GTPases. From Takai *et al.*, Physiol Rev, 2001. **81**(1): p. 153-208.

Regulation of small GTPases function

In addition to the spatial regulation by posttranslational modifications, small GTPases are temporaly regulated by upstream mediators. This is in line with the fact that intrinsically small GTPases are very inefficient at both GTP hydrolysis and nucleotide exchange, Their role as 'molecular switches' in the signaling pathways though, requires rapid alteration between the 'inactive' and 'active' states. Organisms thus have evolved two major groups of proteins that regulate the small GTPases activity by accelearting their transition between the two different states. GEFs (Guanine Nucleotide Exchange Factors) promotes the exchange of GTP for GDP thus activates small GTPases, while GAPs (GTPase Activating Proteins) accelerates the intrinsic GTPase activity of GTP hydrolysis, resulting in inactivation of small GTPases. The activity of GEF or GAP proteins can be regulated by upstream signals via phosphorylation, adaptor proteins or interactions with phospholipids. These signals eventually lead to the activation or inactivation of specific small GTPases [240] (**Figure 1.6**).

GEFs

GEFs bind to inactive small GTPases and reduce their affinity for the bound GDP, reducing the half-life of GDP binding from several hours to several minutes [240]. After releasing the GDP, small GTPases prefer to interact with a new GTP molecule due to the much higher intracellular concentration of GTP than GDP [234]. The majority of GEFs are multidomain proteins, which enable a regulation from upstream signals, including protein protein/lipid interaction, posttranslational modification and binding of

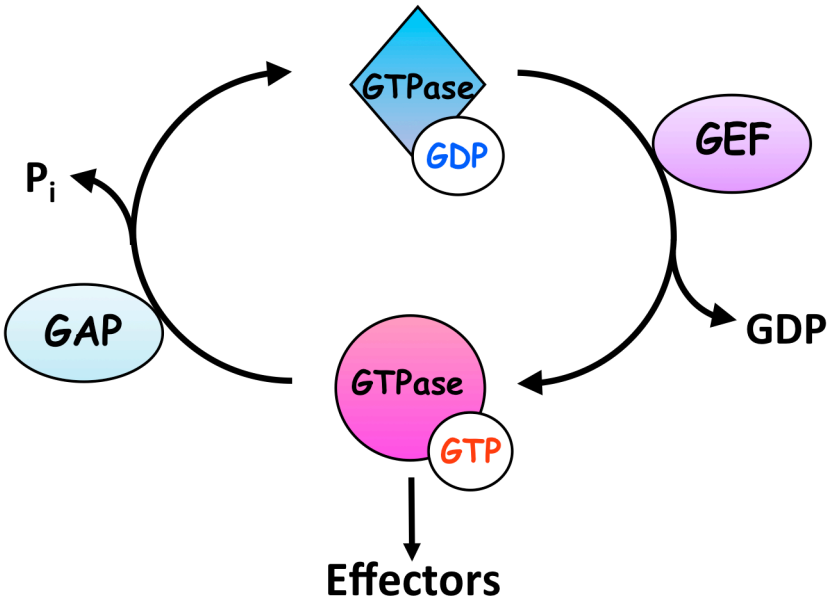

Figure 1.6 Small GTPase cycle and regulation. Small GTPases slowly cycles between the GDP-bound inactive state and the GTP-bound active state. In active states, small GTPases assume a conformation that allows downstream effectors to bind, thus trigger downstream cellular processes. After hydrolyzing the GTP and releasing the inorganic phosphate with their intrinsic enzymatic activity, small GTPases are "switched off" and lose interaction with their effectors. GEFs (guanine nucleotide exchange factors) activate small GTPases by facilitating the exchange from GDP to GTP-binding; GAPs (GTPase-activating proteins) inactivate small GTPases by accelerating the intrinsic GTPase activity of GTP hydrolysis.

second messengers such as Ca++ and diacylglycerol (DAG) [240]. These modifications and interactions usually lead to one or more of three changes on the GEFs activity: 1) a translocation to or away from the intracellular compartments where a target small GTPase resides; 2) a release from or an enhancement of auto-inhibition by an intra-molecular domain that interferes with substrate binding; or 3) an alteration of the catalytic kinetics.

A classical and intriguing paradigm of GEF translocation is through interaction with an upstream small GTPase to access and activate a particular downstream small GTPase. In fact, Ral GTPases are regarded as the first examples of crosstalk between small GTPases, as several RalGEFs contain Ras-binding domains and are under the regulation of activated Ras GTPases [229]. To date, there are at least six different members have been identified in the RalGEF family [241-247], which is subdivided into the RalGDS and RalGPS subfamilies. The RalGDS subfamily members all contain a Ras binding domain (RBD) that interacts with activated Ras GTPases [229, 242, 243, 245, 246]. The RalGDS members act as the effectors of Ras and the regulators of Ral. In response to extracellular signals, such as EGF stimulation, Ras is activated and recruits the downstream effector RalGDS, inducing a translocation that brings the RalGDS in proximity of membrane compartments where it can access and activate Ral [248, 249]. In contrast, the RalGPS subfamily members lack an RBD and may respond to other upstream stimuli than Ras activation, which are still largely unknown [241, 244, 247].

GAPs

GAPs interacts with the active small GTPases and accelerates their rate of GTP hydrolysis by stabilizing the transitional state of the enzymatic reaction [240]. As GEFs, most GAPs contain more than one domain structures and thus are under similar regulation, including protein protein/lipid interaction, posttranslational modification and second messengers binding, which causes GAPs to undergo spatial translocation, change of affinity with substrates or alteration of intrinsic catalytic activity. As discussed previously, in insulin signaling, Akt kinase phosphorylates at least GAP proteins: the Rheb GAP complex TSC1/2, the RalA GAP complex RGC1/2 and a RabGAP AS160. Akt phosphorylation of TSC2, the GAP-domain containing component of the complex, is believed to cause translocation of TSC2 from the plasma membrane to the cytosol, where it is retained by 14-3-3 proteins, thus isolating TSC2 from its functional partner TSC1 and its target small GTPase Rheb [250]. This releases Rheb from constitutive inhibition by TSC1/2 thus the activation of the small GTPase and its downstream effector the mTORC1 complex, eventually promoting cell growth and proliferation [182, 183]. In contrast, Akt phosphorylation on the RGC1/2 is believed to cause a conformational change of the complex that decreases the affinity to its substrate RalA GTPase. Loss of RGC proteins thus leads to increase of RalA activity and consequently enhanced plasma membrane Glut4 levels and increased glucose uptake [97]. Although it is speculated that Akt phosphorylation on the RabGAP AS160 may also negatively regulate its GAP activity towards the target small GTPases, it has not been thoroughly tested and will be the main topic of chapter 2 of this dissertation.

Small GTPases in Glut4 trafficking

The first line of evidence implicating the involvement of small GTPases in Glut4 vesicle trafficking comes from studies showing that incubating adipocytes with non-hydrolyzable form of GTP (GTP-γS) induces Glut4 translocation to the cell surface, mimicking the effect of insulin [251, 252]. Furthermore, some small GPTases have been found localized at Glut4-containing vesicles in adipocytes and cardiac muscle cells [253, 254]. After these initial discoveries, a growing body of literature has revealed the roles of many small GTPases from different subfamilies in regulating various aspects of the Glut4 cycle, including compartmentalization, membrane transport, cytoskeletal recognition, tethering and fusion (**Figure 1.7**). In the following section, I will discuss the studies on these small GTPases in the context of Glut4 vesicle trafficking.

Arfs (ADP-ribosylation factors)

The Arf subfamily of small GTPases regulate vesicle trafficking in multiple processes [255]. One essential role of the active Arf GTPases is to recruit coat proteins to membrane compartments to facilitate cargo sorting, deformation of the donor membrane, and ultimately vesicle budding [256-258]. It is therefore tempting to speculate that one or several Arfs may be involved in regulating the formation of Glut4 storage vesicles. Indeed, Arf6, the best-studied isoform of the Arf subfamily [255], has been implicated in regulating Glut4 trafficking; however, its exact roles and regulatory mechanisms are still controversial. One study observed that introduction of a dominant-inhibitory form of Arf6 into 3T3-L1 adipocytes blocks insulin-induced Glut4 translocation and glucose

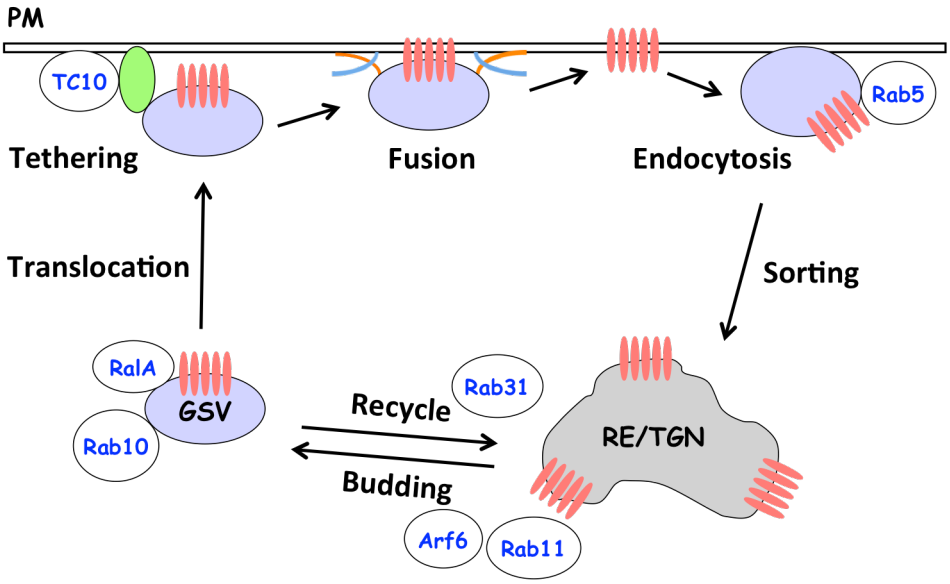

Figure 1.7 Roles of small GTPases in Glut4 trafficking. Glut4 is internalized through Rab5 mediated endocytosis in the absence of insulin. Arf6 and Rab11 are thought to regulate sorting of Glut4 and perhaps generation of Glut4 storage vesicles (GSV) from the recycling endosomes and/or trans-Golgi networks (RE/TGN). Rab31 is believed to promote the futile cycle of GSV back to RE/TGN, thus retaining Glut4 with the intracellular compartments. RalA is associated with GSV and may regulate movement of the vesicles along actin cable as well as the docking of the vesicles at the plasma membrane. TC10 localizes on the plasma membrane, and facilitates Glut4 vesicle docking by regulating exocyst assembly. Rab10 may be involved in Glut4 vesicle transport as well as the distal steps of Glut4 exocytosis including docking and fusion.

uptake [259]; however, other studies with similar approaches revealed a lack of effect of Arf6 in either insulin-stimulated Glut4 translocation or glucose uptake [260-262]. Although additional studies will be required to clarify the exact role of Arf in regulating Glut4 transport, several effector proteins of Arf have been implicated in regulating this process.

One of these effector proteins is the GGA (Golgi-localized γ-ear-containing Arf binding proteins), which is a group of adaptor proteins that are indicated in the biosynthetic sorting of Glut4 from TGN into the GSV (Glut4 Storage Vesicles) [43, 263, 264]. Expression of a dominant-negative form of GGA in adipocytes inhibits insulin-stimulated glucose uptake, which may result from a defect in GSV formation judged by a reduced amount of Glut4 in small vesicles and a concomitantly increased amount of Glut4 in the TGN/RE (trans-Golgi networks/recycling endosomes) [263, 264]. GGA possibly mediates GSV genesis not through directly binding to Glut4 *per se* [143], but by interacting with the major GSV-resided protein sortillin [65], which has recently been shown to be both sufficient and essential to generate GSV [66, 67]. Another Arf effector protein that may mediate Glut4 sorting is ACAP1, which is also a GAP of Arf6 [265]. ACAP-1 interacts with both the coat protein clathrin and Glut4 itself during vesicle formation on the TGN/RE [266]. Indeed, siRNA-mediated knockdown of either ACAP-1 or clathrin heavy chain decreases insulin-stimulated glucose transport, consistent with their role in GSV formation [266]. Additionally, there is evidence that the clathrin adaptor protein AP-1 interacts with Arf on the TGN/RE and participates in GSV genesis, potentially in coordination with GGA [267-269]. These studies, taken together, all

support the idea that Arf GTPases regulate coat assembly of the GSV, as of other intracellular vesicles, via adaptor protein recruitments [255].

Rab

With more than 60 members, the Rab proteins constitute the largest branch of the Ras superfamily GTPases [236]. Numerous studies have shown that Rab GTPases are distributed to and activated at distinct membrane compartments, where they tightly regulate multiple aspects of vesicle transport between organelles [236, 270]. Consistent with their critical role in vesicle trafficking, a number of Rab proteins have been implicated in different steps in insulin-stimulated Glut4 transport [167, 230].

Rab4. Rab4 localizes to the early and recycling endosomes and is thought to regulate sorting and recycling of the endocytosed receptors from this compartment back to the plasma membrane [271, 272]. In adipocytes and muscle cells, Rab4 has been found to be associated with immunopurified Glut4 vesicles, and to redistribute to the plasma membrane in response to insulin stimulation [254, 273-275]. Overexpression of either wild-type or dominant-negative mutants of Rab4 has been shown to block insulin-induced Glut4 translocation [273, 276, 277], making the precise role of Rab4 in this process confusing and unclear. Some recent study suggests a role of the Rab4 and Rab5 effector Rabip4 in Glut4 translocation, although the exact function of this protein is also unknown [278]. Two additional Rab4 effectors have been shown to be involved in Glut4 trafficking. One is the motor protein KIF3, which is recruited by the active Rab4 and mediates microtubule movement of GSV towards the plasma membrane [38]. The GTP-

bound Rab4 also exhibits a direct interaction with Syntaxin-4 [279], which is the t-SNARE protein required for GSV fusion with the plasma membrane [64, 107-109]. Together, these studies suggest a role of Rab4 in regulating GSV translocation and fusion.

Rab5. Rab5 is a key regulator of multiple membrane endocytic events, including formation of clathrin-coated endocytic vesicles [280], motility and fusion of early endosomes [281, 282], and turnover of phospholipids [283, 284]. Although insulin regulates both the activity and the localization of this GTPase [285, 286], the roles of Rab5 in Glut4 trafficking are contradictory. Studies have suggested that Rab5 regulates both the Glut4 endocytosis and insulin-stimulated Glut4 exocytosis. Microinjection of anti-Rab5 antibody into 3T3-L1 adipocytes inhibits the internalization of plasma membrane-inserted Glut4 [285], suggesting Rab5 decreases Glut4 membrane localization by enhancing the endocytosis. In contrast, overexpression of dominant-negative Rab5 blocks adiponectin-stimulated Glut4 translocation [287], indicating a positive role of Rab5 in Glut4 membrane translocation. Rab5 is reported to regulate Glut4 exocytosis through one of its effectors APPL1, which is an adaptor protein that binds to adiponectin receptor and Akt kinase [287, 288]. Upon activation by adiponectin, Rab5 recruits APPL1 to the plasma membrane and facilitates Glut4 translocation [287]. Furthermore, Rab5 directly interacts with and stimulates the activity of PI-4 and PI-5 phosphatases [284], resulting in increased PI3P formation at the plasma membrane, which is potentially important for insulin-stimulated Glut4 trafficking [118, 209-211]. Hence Rab5 appears to play contradictory roles in regulating Glut4 translocation. While the reason for this phenomenon is still unclear, it is possible that multiple pools of Rab5 are required in the

cells to achieve a refined temporal and spatial regulation on Glut4 trafficking. Future studies to reveal these diverse functions of Rab5 in insulin action are therefore of high interest.

Rab11. Rab11 has been implicated in regulating recycling endosome to TGN transport, as well trafficking of recycling endosome to the plasma membrane [289, 290]. Fractionation and proteomic analysis has found that Rab11 is associated with GSV [62, 179, 291]. Some studies also reveal that Rab11 undergoes translocation with the GSV to the plasma membrane in response to insulin [291]. Similar to Rab4, the requirement of Rab11 in insulin action remains controversial, since overexpression of wild-type or dominant negative Rab11 in muscle cells exhibits inhibitory effect on insulin-stimulated Glut4 translocation and glucose uptake [292]. However, in adipocytes, it has been shown that inhibition of Rab11 function blocks both basal and insulin-stimulated Glut4 exocytosis, likely due to the decreased formation of GSV, indicating that Rab11 is required for the sorting of Glut4 from recycling endosomes to the specialized GSV [293]. Furthermore, in adipocytes, Rab11 may also regulate the production of PI3P [294], a lipid that is generated at the plasma membrane in response to insulin and required for insulin-stimulated glucose uptake [118, 209-211].

Rab8A/10/13, Rab14. Rab8A, Rab8B, 10 and 13 constitute a subfamily of mammalian Rab small GTPases that are closest to the yeast SEC4, an essential gene product involved in post-Golgi constitutive secretion [295]. Early studies intended to pinpoint the

subcellular localization of these Rab small GTPases found that despite their high sequence homology, they localize to different intracellular compartments, potentially due to the variations in their C-terminal targeting motifs. While Rab8 is found at the cell periphery with the highest concentration in filopodia and ruffles, localizes on membranes in perinuclear region overlapped with the Glogi markers [295, 296]. In contrast, Rab13 resides in the perinuclear region and in cell-cell contacts, where junctional complexes developed with neighboring cells [297]. Intriguingly, to date, Rab8, Rab10 and Rab13 are found to regulate distinct cellular processes that may be largely accounted for by their subcellular localizations. For instance, by regulating the formation of the dynamic structures like filopodia, lamellipodia and ruffles, Rab8 has a strong influence on cell morphogenesis, cell polarity, migration and ciliogenesis [296]. Rab10, instead, is found to regulate multiple vesicle trafficking processes such as biosynthetic transport to basolateral membrane, Na/K/ATPase translocation to plasma membrane, and soluble extracellular protein release [298-300]. Consistent with its localization to the junctional complexes, Rab13 plays a critical role in regulating the tight junction assembly in epithelial cells [297, 301, 302].

Rab 8A/10/13 and Rab14 have recently gained special attention because of the identification of a RabGAP AS160 [71, 168]. In an effort to identify novel Akt substrates in adipocytes, Gustav Lienhard and coworkers used mass spectrometry to analyze phosphorylated proteins immunoprecipitated from mock-treated or insulin stimulated cells with the phospho-Akt substrate antibody [168]. A 160-kDa phosphorylated protein with a RabGAP domain at its C-terminus was found in this screen, which is named

AS160 (Akt Substrate of 160 kDa) [168]. Further studies established a regulatory role of AS160 in insulin-stimulated Glut4 translocation in 3T3-L1 adipocytes and L6 myoblasts [71, 176]. Furthermore, incubation of the C-terminal GAP region of AS160 with 18 recombinant Rab GTPases found that this GAP domain is able to accelerate the *in vitro* GTP hydrolysis of Rab2, Rab8A, Rab10 and Rab14 (Rab13 was not screened in this study) [72]. In addition, in a comprehensive proteomic analysis of affinity-purified Glut4 vesicles from 3T3-L1 adipocytes, Rab10 and Rab14 are found to be associated with Glut4 vesicles, as is AS160 [72, 179]. These findings have raised a great interest on understanding the role of these Rab GTPases in Glut4 trafficking.

Among the potential AS160 substrates, Rab2 is less likely a candidate to regulate Glut4 translocation, as there is strong evidence that it regulates trafficking between the endoplasmic reticulum and the Golgi [72, 303]. Further investigation has therefore focused on the role of the Rab8A/10/13 subfamily and Rab14 in insulin stimulated Glut4 translocation. Depletion of these Rab GTPases with siRNA in adipocytes and muscle cells revealed surprising yet intriguing results. While knockdown of Rab10 in 3T3-L1 adipocytes significantly decreases insulin-induced Glut4 translocation, neither knockdown of Rab8A nor Rab14 has exhibited any effect in these cells [74, 76]. In contrast, in L6 muscle cells, depletion of Rab8A, Rab13, or Rab14 all result in impaired Glut4 surface exposure in response to insulin, whereas knockdown of Rab10 shows little effect in this cell type [73, 75]. The underlying mechanism of this apparent cell-type specific requirement of Rab GTPases in regulating Glut4 exocytosis remains to be established. One possible explanation is that Glut4 resides in different intracellular

compartments associating with different Rab GTPases, while the extents of these compartments may differ in the different cell lines used in these studies [171]. It is also possible that despite belonging to the same subfamily, Rab8A, Rab10 and Rab13 may actually answer to different upstream signals and engage distinct downstream machinery effectors to facilitate Glut4 translocation in adipocytes and muscle cells. Indeed, one study suggests that the molecular motor myosin Vb may be an effector of Rab8A leading to Glut4 mobilization in muscle cells [72]. Interestingly, other studies indicate that while both Rab8A and Rab10 can interact with myosin Vb, the interaction with Rab10 but not Rab8A is dependent on the presence of an alternatively spiced exon D in the myosin Vb molecule [304, 305]. It is therefore tempting to speculate that in muscle cells, myosin Vb is the downstream effector of Rab8A but not Rab10 in mobilizing Glut4 translocation, since in these cells the predominantly expressed myosin Vb isoform is without the exon D [304]. Nonetheless, there is still very little known about the downstream effectors of Rab8A or Rab10 in the context of Glut4 traffic, further studies in this regard are therefore of great interest and will unravel the regulatory role of these small GTPases in Glut4 translocation. In chapter 3, I will present data suggesting the RalA GTPases as an indirect downstream effector of Rab10 in regulating Glut4 trafficking.

Rab31. Rab31 colocalizes with markers of endosomes and TGN [306], and has been implicated in regulating TGN to endosome transport [81]. Rab31 is a substrate of Gapex-5, which contains a VPS9 domain that facilitates the GTP-binding of Rab31 [80]. It is thought that Gapex-5 maintains Rab31 in an active state in the absence of insulin [80], which is consistent with other observations showing that Rab31 has high constitutive

levels of GTP loading in the basal state [306, 307]. Insulin stimulation decreases Rab31 activity by recruiting Gapex-5 to the plasma membrane. Furthermore, overexpression of wild-type or constitutively active Rab31 inhibits insulin-stimulated Glut4 translocation, whereas knockdown of Rab31 enhances insulin-simulated Glut4 translocation and glucose uptake [80]. Together, these data suggest a model in which active Rab31 retains Glut4 vesicles within the cytosol in the basal state; while insulin stimulation inactivates Rab31, thus permitting Glut4 vesicles to translocate to the cell surface [80]. However, the mechanism of this intracellular retention is still unclear, therefore looking for the Rab31 effectors involved in insulin action and Glut4 translocation will be of great interest.

Ras

Ras. Although Ras has long been regarded as an important player involved in the growth-promoting effects of insulin, its role in metabolic actions of insulin is controversial [224-227]. Upon activation as downstream of the insulin receptor, Ras initiates a series of phosphorylation events leading to the phosphorylation and activation of MAPK by the upstream MAPK kinase MEK [222]. Previous studies with inhibitors targeting this signaling pathway indicate that Ras has little, if any, impact on insulin-stimulated Glut4 translocation [226, 228]. Furthermore, overexpression of either the constitutively active Ras or the dominant negative Ras in adipocytes shows little effect on Glut4 trafficking, although Ras activation seems to increase glucose uptake by upregulating the expression of another glucose transporter, Glut1 [225, 308, 309]. However, *in vivo* studies reveal that transgenic overexpression of active Ras in mice adipose tissue improves insulin sensitivity, although the increased glucose uptake in these mice may be partially

accounted for by the upregulated Glut1 level [227]. Despite current evidence suggesting against the involvement of Ras in insulin-stimulated Glut4 translocation, it is noteworthy that activation of Ras may increase the activity of other small GTPases such as Ral and Rab5 through regulating their GEFs [310, 311], both of which are known mediators in Glut4 trafficking. Therefore, further studies regarding the crosstalks between Ras and other small GTPases may reveal interesting roles of Ras in insulin action and Glut4 trafficking.

RalA. Ral (Ras-like) small GTPases (RalA and RalB) are members of the Ras subfamily [312], and have been implicated in varies cellular processes including control of gene expression, cell motility and polarity, and protein trafficking [312-314]. The two Ral isoforms share 85% identical sequences, with the most variation in the C-terminal targeting motif, which contributes to the distinct localizations of RalA and RalB in cells [96, 315, 316]. While RalB has been shown to primarily present on the plasma membrane, RalA has been found on both the plasma membrane and many intercellular vesicular structures including synaptic vesicles, platelet granules as well as Glut4 vesicles [36, 317, 318]. Consistent with the different subcellular localizations, the functions of RalA and RalB are not completely overlapping [98, 319, 320]. For instance, while both RalA and RalB can interact with two components of the exocyst complex, Exo84 and Sec5 [98, 99, 321], RalA has higher affinity than does RalB [96], indicating that RalA may play a bigger role in exocyst function, such as the establishment of cell polarity [98], and basolateral membrane trafficking in epithelial cells [96].

Recent studies have established an important role of RalA in regulating insulin-stimulated Glut4 trafficking. Depletion of RalA, Exo84 or Sec5 with siRNA-mediated knockdown blocks insulin-stimulated Glut4 translocation and glucose uptake in adipocytes [36]. Moreover, overexpression of RalA mutants that cannot bind to the exocyst components causes inhibition on Glut4 exocytosis, suggesting the exocyst as the major effector responsible for RalA in regulation of Glut4 trafficking [36]. Upon activation by insulin, RalA is believed to unify the vesicle-localized exocyst subunits such as Exo84, with the plasma membrane associated exocyst subunits such as Sec5 [99, 322]. This facilitates the assembly and function of the full exocyst complex, which targets the Glut4 vesicles to the plasma membrane. The exocyst complex is then disengaged from the Glut4 vesicles though insulin-stimulated, PKC-dependent phosphorylation of Sec5, a step required for the final fusion of the Glut4 vesicles with the plasma membrane [39]. As discussed earlier, RalA also regulates Glut4 translocation through its interaction with Myo1c [36], a myosin motor that plays an important role in translocating the Glut4 vesicles towards the plasma membrane along the actin fibers.

RalA is rapidly activated by insulin in a PI 3-kinase dependent manner in adipocytes [36]. Further studies identified a RalA GAP complex RGC1/2 that connects the insulin/PI 3-Kinase signaling to RalA. RGC1/2 contains several Akt kinase phosphorylation sites and a GAP that catalytically inactivates RalA at the basal state. In response to insulin, Akt directly phosphorylates RGC proteins; this process is believed to result in a conformational modification of RGC1/2 that decreases the interaction between the

complex and RalA. Loss of RGC proteins thus leads to increase of RalA activity and consequently enhanced plasma membrane Glut4 levels and increased glucose uptake [97].

Although the RGC1/2 complex plays a critical role in regulating insulin-stimulated RalA activation in adipocytes, it is possible that other regulatory mechanisms may exist. As discussed before, several RalGEFs contain Ras-binding domains and act as the effectors of Ras and the regulators of Ral. As Ras is activated by many extracellular signals, such as EGF (epidermal growth factor), it is possible that RalA may be also activated by these signals through the bridging of the RalGEFs and may participate in Ras-regulated cellular processes. This idea was supported by studies indicating a critical role of RalGEFs in regulating Ras-mediated *Drosophila* eye development [323] and promoting Ras-dependent transformation and tumorigenic growth of human cells [313, 324, 325]. It is therefore of great interest to see whether RalA is activated by insulin through the Ras-RalGEF pathway, and whether this pathway participates in mediating the insulin-stimulated Glut4 translocation. It is also intriguing to see whether other links exist that bridge insulin action and RalA activation particularly through regulations on RalGEFs proteins.

Extensive studies would be required to unravel the above mentioned mechanisms, particularly as there are multiple RalGEF members playing diverse roles in different cellular processes and cell types, and all contain a variety of regulatory domains that respond to different upstream signaling [229]. Nevertheless, using the combination of proteomic approaches and a specific RalA mutant that preferentially interacts with GEF

proteins, our lab has found a known Ras effector and RalGEF protein, Rlf, strongly associating with RalA in COS cells (Chen and Saltiel, unpublished data). However, follow-up studies are needed to fully understand the function of Rlf and the Ras-Rlf-RalA axis in the context of insulin regulated Glut4 translocation.

Rho

Rho/Rac/Cdc42. The Rho family GTPases, including isoforms of Rho, Rac and Cdc42 have been well characterized for their role in regulating actin rearrangement, a process critical for driving a variety of cellular processes from vesicle transport events to cell morphology and polarity [326, 327]. Inhibitors disrupting actin filaments largely inhibit insulin-stimulated Glut4 translocation and glucose uptake [88, 89], suggesting an important role of actin remodeling in Glut4 trafficking. Many studies therefore focused on the possible role of Rho family small GTPases in insulin-stimulated Glut4 translocation, which however obtained conflicting findings. While inactivating Rho/Rac with clostridia toxins has been shown to inhibit insulin-stimulated glucose transport in 3T3-L1 adipocytes [328], overexpression of mutant forms of Rho and Rac have led to inconsistent results in these cells [204, 329, 330]. Additionally, the action of Cdc42 in insulin signaling is also unclear. One study has shown that depletion of Cdc42 with microinjection of anti-Cdc42 antibodies or siRNA knockdown causes decreased insulin-stimulated Glut4 translocation in adipoctyes [331], while another study has reported no effect of Cdc42 mutants on Glut4 translocation or glucose uptake in these cells [204]. Although the cause of the discrepancy between these studies remain unclear, it should be noted that studies with mutant forms of GTPases need to find the optimal expression

levels of these mutants to avoid potential toxicity and to achieve desired biological effects.

TC10. TC10 α and β are mamalian Rho family small GTPases that are most similar in sequence to Cdc42 [205]. The effector binding domains fo TC10 and Cdc42 are highly conserved, which is the structural basis of these two small GTPases sharing many common effectors including the N-WASP, CIP4, the Par3/6 complex and PAKs (p-21 activated kinases) [208, 213, 332-334]. Despite the high similarity, several lines of evidence suggest that TC10 and Cdc42 are not functionally redudant, but rather play disinct roles in regulating different intracellular events. First, unlike Cdc42, no homologue of TC10 has been identified in yeast, and expression of human TC10 in a yeast *cdc42* mutant fails to rescue the phenotype like expression of the mammalian Cdc42 [335]. Furthermore, none of the many Cdc42 GEFs or extracellular stimuli known to activate Cdc42 is able to promote the activation of TC10, indicating that the activity of these two small GTPases are differently regulated [336]. More importantly, while Cdc42 as well as other Rho family small GTPases undergo geranylgeranylation at their C-terminal targeting motif [231], TC10 has a tandem palmitoylation and farnesylation at this region [337, 338]. The different lipid modifications at least partially account for their differences in subcellular localization—TC10 is largely found in lipid raft microdomains in the plasma membrane and endosomes [335, 337], wherease Cdc42 is localized mainly to a perinuclear region [335, 336]. Taken together, these data suggest that TC10 and Cdc42 have distinct functions in cells.

Several studies support an important role for TC10 in insulin-stimulated glucose transport. For example, both TC10 isoforms are highly expressed in insulin-responsive tissues and are rapidly activated upon insulin stimulation [204, 205]. Overexpression of the dominant negative form of TC10α but not β is shown to block insulin-stimulated Glut4 translocation and glucose tuptake, suggesting the α isoform primarily functions in adipocyte to mediate insulin action [204, 205]. Consistent with this, knockdown of TC10α in adipocytes also leads to impaired Glut4 translocation and glucose uptake in response to insulin [339]. As discussed in details before, upon activation, TC10 recruits multiple effectors that are implicated in regulating Glut4 exocytosis, including an adaptor protein CIP4, Par6 of the Par3/6 complex and the Exo70 subunit of the exocyst complex [28].

IV. Summary

More than two decades after the role of Glut4 in insulin-stimulated glucose uptake in muscle and adipose tissue was described, our understanding of the mechanisms by which insulin regulates the transporter's function remains incomplete. Substantial evidence now suggests that the 'fine tuning' of insulin-regulated Glut4 trafficking is achieved through a complex network of signaling pathways and transport machineries. The PI 3-kinase dependent and independent pathways likely mobilize different downstream proteins but may also converge on certain targets such as the one of the trafficking machineries, the exocyst complex. The small GTPases are regarded as critical intermediates that connect

the signaling events and the transport machineries in Glut4 exocytosis. The activity of these small GTPases is tightly regulated by their mediators GEFs and GAPs in response to signaling input from the insulin receptors. To date, there are several examples of GEFs (C3G, Gapex-5) and GAPs (RGC1/2, AS160) implicated as targets of insulin in regulating small GTPase activity. However, we do not yet fully understand how insulin regulates these proteins or their roles in Glut4 trafficking. The focus of my dissertation work has been to characterize the regulation of the activity of some of these small GTPases. In chapter 2 of this dissertation, I will establish Rab10 as a direct *in vivo* target of the AS160 GAP activity through a novel assay; I will also demonstrate that phosphorylation of AS160 by Akt negatively regulates its GAP activity towards Rab10. In chapter 3, I will present data suggesting that RalA as an indirect downstream effector of Rab10 in regulating Glut4 trafficking.

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