Chapter 2

Characterization of AS160 activity and its regulation by Akt

Introduction

Insulin stimulated glucose transport plays a central role in maintaining whole-body

glucose homeostasis [1, 2]. The hormone increases glucose disposal into adipose and

muscle tissue through a process of regulated vesicle trafficking, in which the facilitative

glucose transporter Glut4 is recruited from the intracellular storage vesicles to the plasma

membrane [2-4]. Despite much attention, our understanding of the insulin signaling

cascades governing this process remains incomplete [4, 5]. Nevertheless, activation of the

protein kinase Akt downstream of PI 3-kinase has been regarded as the key signaling

event in insulin-stimulated Glut4 translocation [4, 6, 7], although the exact mechanisms

by which Akt signals downstream targets involved in the transport process remain to be

fully elucidated.

Recent studies have suggested the family of small GTPases as one possible target of the

Akt signaling pathway in regulating Glut4 trafficking [5, 8, 9]. Small GTPases are

regarded as a group of "molecular switches" that connect signaling events with vesicle

transport machineries [10, 11] by alternating between the GTP-bound active state and

GDP-bound inactive state. Upon activation, small GTPases are able to recruit

downstream effectors to facilitate vesicle trafficking, such as the cytoskeleton-regulating

kinases, molecular motor proteins and membrane fusion proteins [12, 13]. The activation

of small GTPases is mainly determined by two groups of molecules: GEFs (guanine nucleotide exchange factors) that promote GTP-binding and activate small GTPases, and GAPs (GTPase activating proteins) that accelerate GTP-hydrolysis and inactivate small GTPases [14]. GEFs and GAPs are tightly regulated by upstream signaling inputs through post-translational modification or protein protein/lipid interaction [14]. One example is the RGC1/2 complex, a newly identified GAP complex that inactivates the small GTPase RalA and plays an important role in regulating insulin-stimulated Glut4 translocation [15, 16]. The RGC1/2 complex is directly phosphorylated by Akt in response to insulin, resulting in reduced GAP activity and relieving the RalA GTPase from inactivation [16]. Activated RalA promotes Glut4 vesicle translocation to the plasma membrane by engaging downstream effectors, including the exocyst complex and the myosin motor Myo1c [15].

Another Akt substrate that has been implicated in regulating Glut4 tranlsocation is AS160 [17, 18], which contains multiple Akt phosphorylation sites and a RabGAP domain at its C-terminus [19]. *In vitro* GTP hydrolysis assays revealed that the recombinant AS160 GAP domain selectively accelerates GTP hydrolysis of Rab2, Rab8A, Rab10 and Rab14 [20]. Further studies revealed that Rab10 but not Rab8A or Rab14 is required for insulin-induced Glut4 translocation in 3T3-L1 adipocytes [21, 22], while in L6 muscle cell lines, Rab8A and Rab14 but not Rab10 appear to be necessary for Glut4 translocation [23]. Despite being implicated in many studies, however, there is currently no direct evidence that any of these Rab GTPases are the functional targets of AS160 in vivo. It is still unclear whether phosphorylation of AS160 by Akt is related to Rab target activation.

Here we establish Rab10 as a functional target of the AS160 in the insulin signaling, and demonstrate that the GAP activity of AS160 towards Rab10 is negatively regulated by Akt phosphorylation.

Results

Full-length AS160 interacts with Rab10 in a transition state-dependent manner

To detect the *in vitro* GAP activity of the full-length AS160, a pull-down assay was developed, as described before [16]. Previous studies have demonstrated that GAP proteins preferentially interact with their cognate small GTPases during the transition state of GTP hydrolysis [14, 24], the point when GTP is attacked by an H_2O molecule, but has not yet released the γ-phosphate. Although the transition state is highly transient, studies have shown that GDP in complex with aluminum fluoride (GDP/AIFx) resembles the conformation of a GTP molecule during the transition state (GDP--Pi). This chemical complex can thus stabilize the GAP-GTPase interaction by mimicking the reaction intermediate without allowing for catalysis and release [24, 25] **(Figure 2.1A)**. We took advantage of this property and developed a GDP/AIFx-loaded GTPase pull-down assay to evaluate the interaction between full-length AS160 and several small GTPases in the transition state. Because, Rab10 has been implicated as an AS160 target, immobilized glutathione S-transferase (GST)-Rab10 was loaded with GDP/AIFx to mimic the transition state interaction, and then incubated with COS-1 cell lysates expressing AS160.
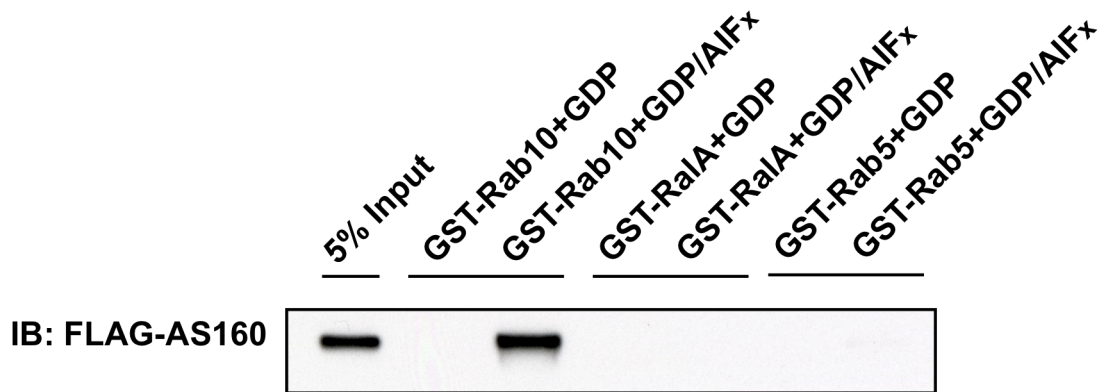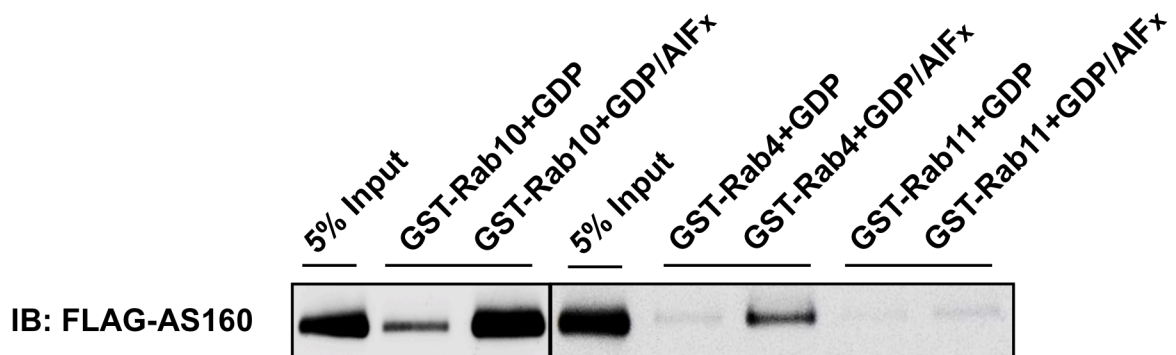
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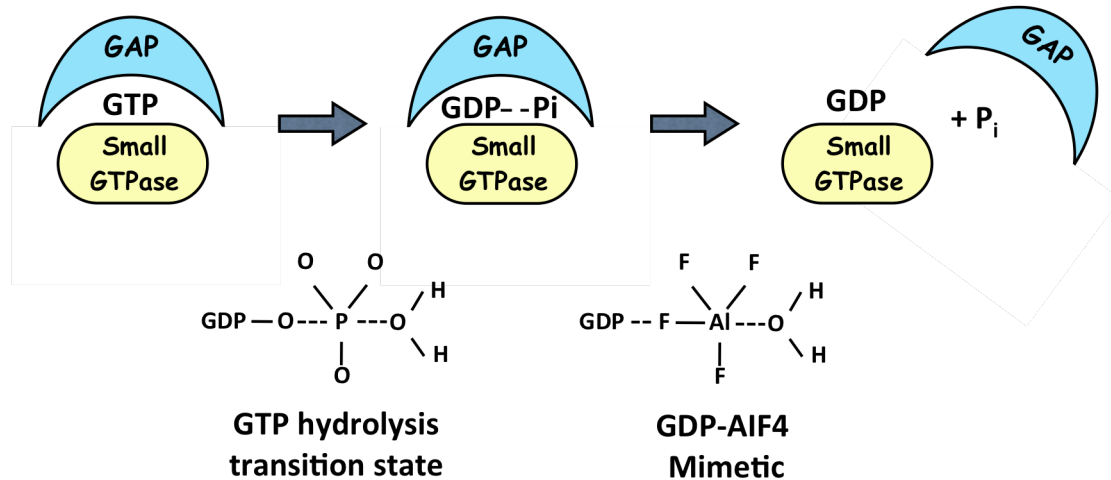
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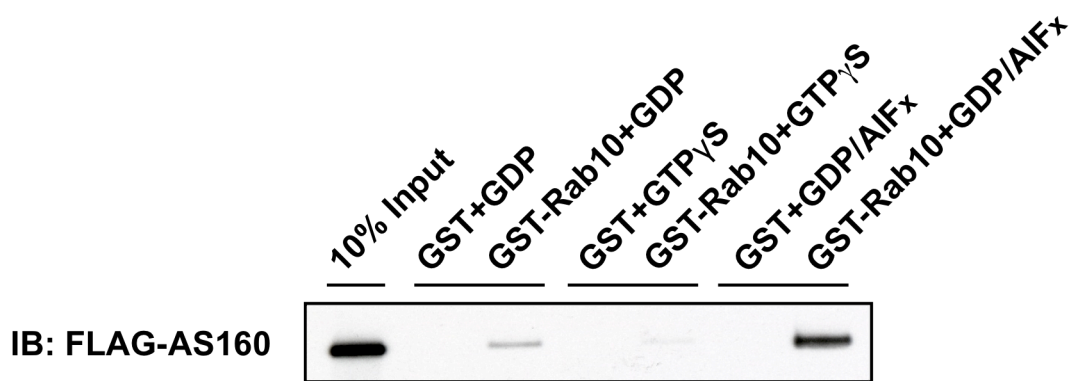
Figure 2.1: AS160 preferentially interacts with Rab10 in the transition state.
A) Schematic view of the interaction between GAPs and cognate small GTPases in the transition state. Top: GAPs preferentially interact with their cognate small GTPases during the GTP hydrolyzing transition state. Bottom: GDP in complex with aluminum fluoride (AIFx) mimics the transition state of GTP hydrolysis. B) COS-1 cell lysate overexpressing FLAG-AS160 was incubated with immobilized GST or GST-Rab10 bound to GDP, GTPγS or GDP/AIFx as indicated. Pull-downs were subjected to SDS-PAGE and Western Blot (WB) with anti-FLAG antibody.

C

IB: FLAG-AS160

D

IB: FLAG-AS160

Figure 2.1: AS160 preferentially interacts with Rab10 in the transition state (continued). C) and D) COS-1 cell lysates overexpressing FLAG-AS160 were incubated with immobilized GST-Rab10, GST-RalA, GST-Rab5, GST-Rab4 or GST-Rab11 bound to GDP or GDP/AIFx as indicated. Associated proteins were separated by SDS-PAGE and detected by WB with anti-FLAG antibody.

As shown in **Figure 2.1B**, AS160 specifically interacted with GST-Rab10 bound to GDP/AIFx, but not with GST-Rab10 bound to GDP or GTPγS, indicating that the interaction between AS160 and Rab10 is transition state-dependent. Meanwhile, AS160 did not interact, or interacted only modestly with other GDP/AIFx loaded small GTPases, including the Ras subfamily member such as RalA and the other Rab subfamily members including Rab4, Rab5 and Rab11 **(Figure 2.1C and D)**. These results suggest that the transition state-dependent binding between AS160 and Rab10 is specific and characteristic of a GAP-substrate GTPase interaction, and further that the full-length AS160 may possess specific GAP activity towards Rab10 *in vitro*.

A GST-Rim1 RBD pull-down assay for assay of Rab10 activity *in vivo*
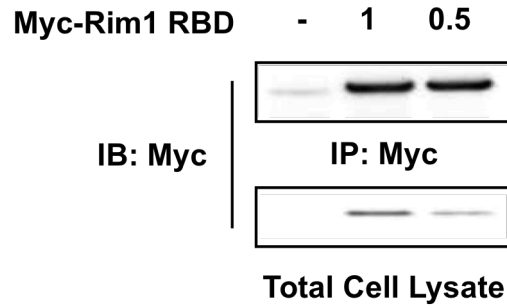
Effector proteins selectively interact with GTP-bound active small GTPases, and have low affinity with the GDP-bound, inactive form. An effector pull-down assay takes advantage of this feature by using an immobilized effector protein to determine the activation status of its cognate small GTPase. As depicted in **Figure 2.2A**, a GST-Rab10-Effector fusion protein was conjugated to glutathione-coupled beads. The amount of Rab10 in the GTP-bound active state was determined by eluting and subjecting the pulled-down proteins to SDS-PAGE and western blot. Previous studies have described similar pull-down assays as effective tools in evaluating the *in vivo* activity of small GTPases including Rab31 and RalA, with their respective effectors EEA1 and RalBP1 [15, 26].
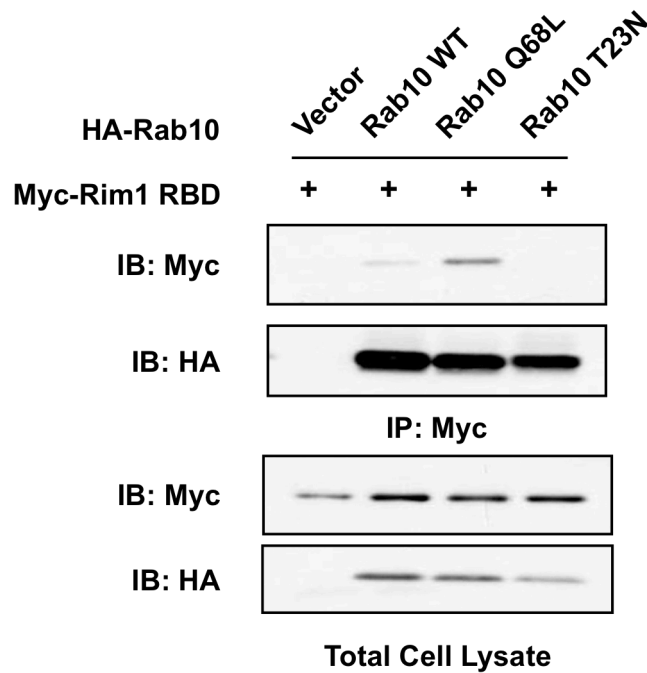
A
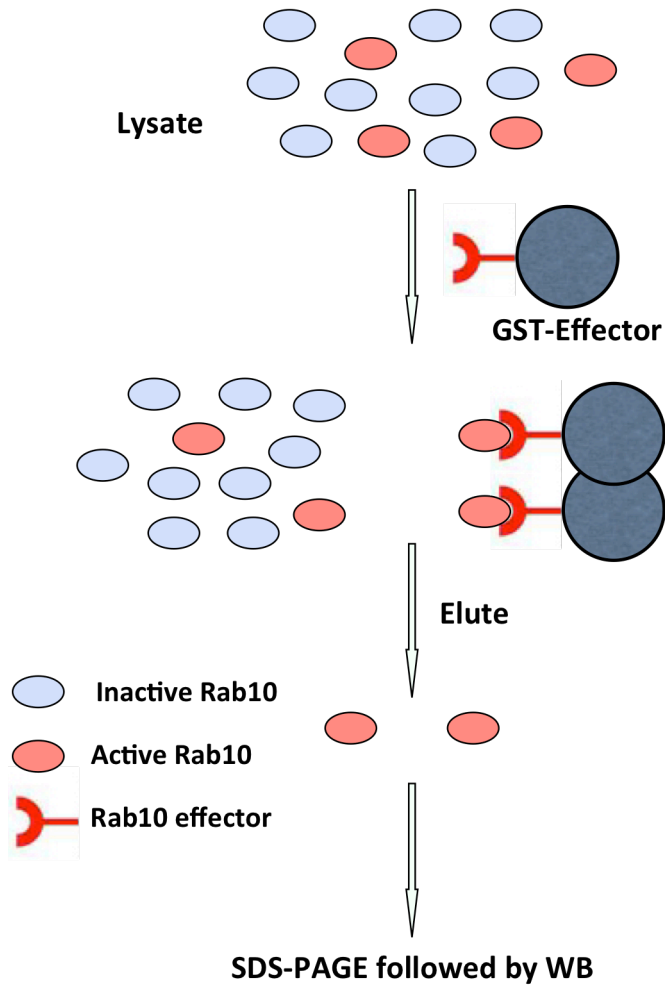
Figure 2.2 Development of effector pull-down assay for Rab10 activity. A) Schematic of effector pull-down assay. Immobilized Rab10 effector selectively isolates the GTP-bound active Rab10 from whole cell lysate. By SDS-PAGE and WB (western blot), the amount and portion of active Rab10 can be estimated.

B

Myc-Rim1 RBD   -   1   0.5

IB: Myc | **IP: Myc**

Total Cell Lysate

C

HA-Rab10   Vector   Rab10 WT   Rab10 Q68L   Rab10 T23N

Myc-Rim1 RBD   +   +   +   +

IB: Myc

IB: HA

IP: Myc

IB: Myc

IB: HA

Total Cell Lysate

Figure 2.2 Development of effector pull-down assay for Rab10 activity (continued). B) Western blot analysis of Myc-Rim1 RBD expression in COS-1 cells. Cells were transiently transfected with 0, 0.5 and 1 μg Myc-Rim1 RBD construct as indicated and cell lysates were subjected to anti-Myc immunoprecipitation and immunoblotting. C) COS-1 cells were transiently transfected with Myc-Rim1 RBD and/or HA-Rab10 WT, Q68L, T23N as indicated. Whole cell lysates (lower panels) or anti-Myc immunoprecipitates were subjected to immunoblotting with anti-Myc or anti-HA antibodies.
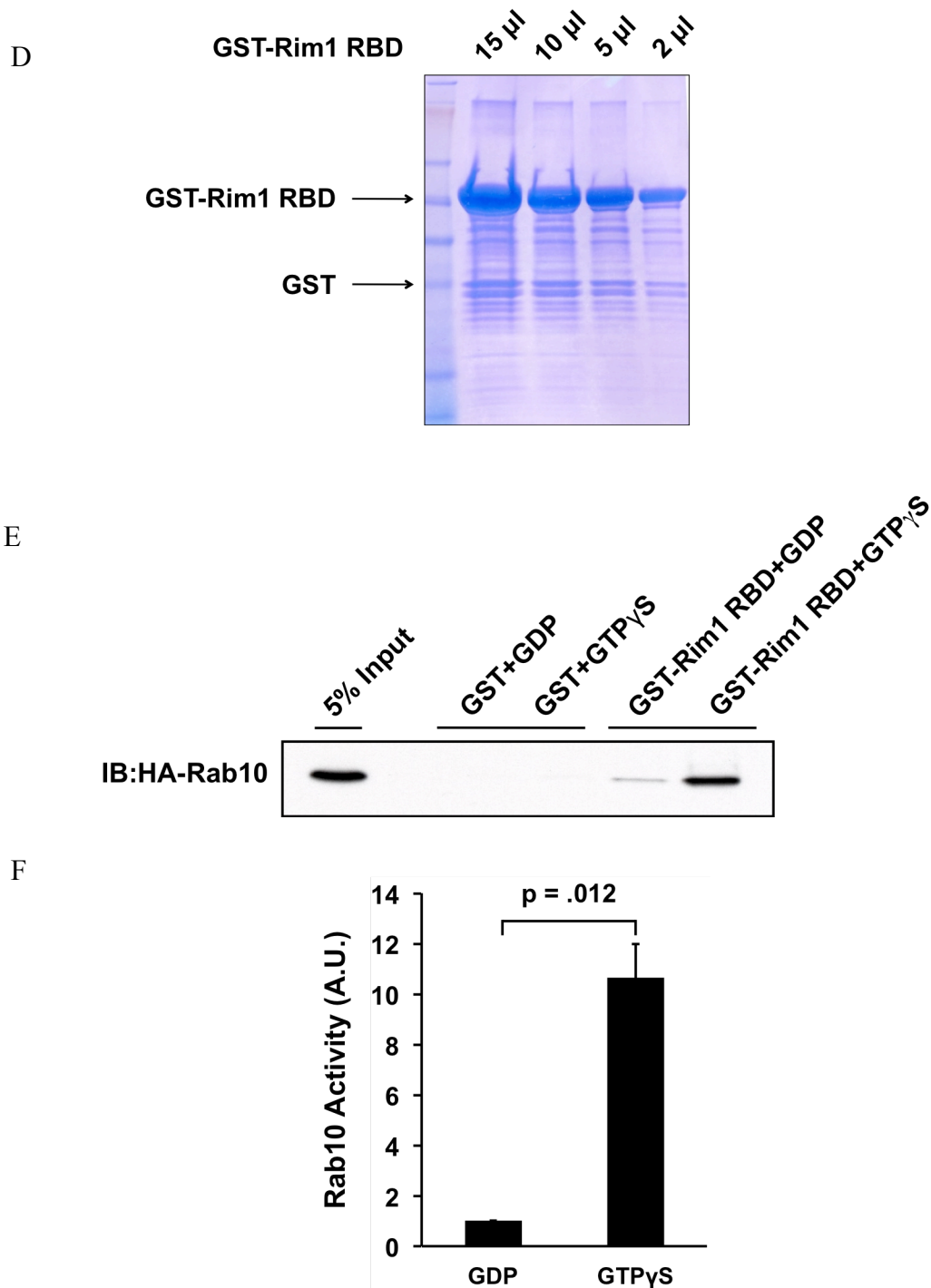
Figure 2.2 Development of effector pull-down assay for Rab10 activity (continued).
D) Coomassie blue staining of GST-Rim1 RBD. E) COS-1 cells were transiently
transfected with HA-Rab10 construct and cell lysates were incubated with immobilized
GST or GST-Rim1 RBD bound to GDP or GTP γS as indicated. Associated proteins
were resolved on SDS-PAGE and determined by Western Blot with anti-HA antibody.
F) Quantification of GDP or GTP-bound Rab10 isolated by GST-Rim1 RBD from three
independent experiments. In each experiment, the amount of isolated GDP-Rab10 was
set as one arbitrary unit. Significance was determined by Student's *t* test.

G
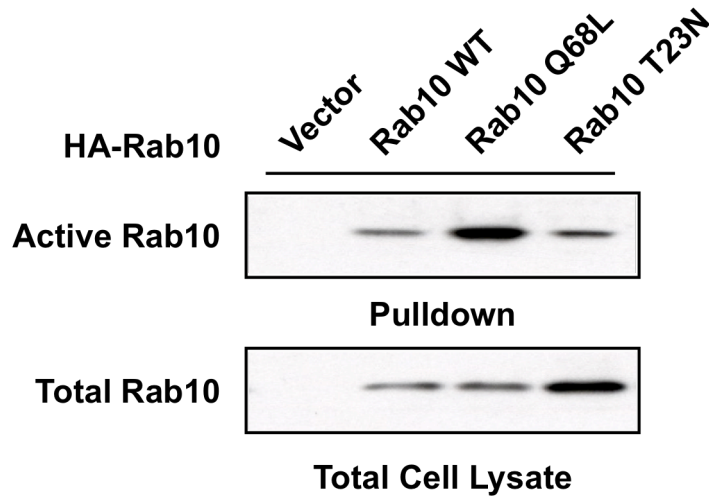
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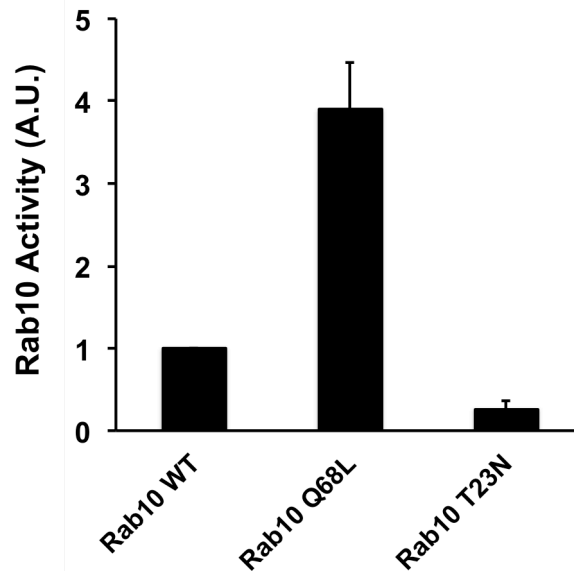
Figure 2.2 Development of effector pull-down assay for Rab10 activity (continued). G) COS-1 cells were transiently transfected with HA-Rab10 WT, Q68L or T23N constructs as indicated and cell lysates were incubated with immobilized GST-Rim1 RBD. Pull-downs were resolved on SDS-PAGE and determined by Western Blot with anti-HA antibody. Lysates represent 2% of total protein from a 10-cm plate of COS-1 cells. H) Quantification of Rab10 activity from two independent experiments. In each experiment, Rab10 activity was quantified by normalizing the pull-down with the total cell lysate. The value of the activity of wild-type Rab10 was then set as one arbitrary unit.

In order to establish a pull-down assay for Rab10, we first searched the literature for potential Rab10 effector proteins. Rim1 (Rab3 Interacting Molecule 1) was reported to interact with Rab10 through the Rab-binding domain (RBD) at its N-terminus [27], though it is unknown whether this interaction is GTP-dependent. In addition, Rim1 appears to have low affinity for the other two Rab10 subfamily members Rab8A and Rab13 [27], rendering Rim1 a possible candidate for the pull-down assay.

To determine whether Rim1 is a Rab10 effector, we characterized the interaction between Rim1 and Rab10 in different nucleotide-binding states. Firstly, the N terminal Rab-binding domain (RBD) was subcloned into a myc-tag vector and the expression of this construct was tested in COS-1 cells (**Figure 2.2B**). We then examined the interaction between myc-Rim1 RBD with the wildtype (WT), constitutively active ($Q^{68}L$) [21] or constitutively inactive ($T^{23}N$) [28] Rab10 via co-immunoprecipitation. In general, substitution of a critical glutamine with a leucine (QL mutation) disrupts the intrinsic GTPase activity in Rab GTPases, thus causing an accumulation of the GTP-bound form of the Rab proteins [21, 29]. Conversely, mutating a key threonine in the GTP-binding domain to asparagine (TN mutation) decreases the affinity of the Rab GTPases for GTP, leading to an increase of a GDP-bound form [28, 30-32]. COS-1 cells were transfected with myc-Rim1 RBD alone or together with HA-tagged Rab10 WT, Q68L or T23N, and lysates were subjected to co-immunoprecipitation. As indicated in **Figure 2.2C**, Rim1 RBD interacted with the Rab10 $Q^{68}L$ mutant with the highest affinity, but exhibited a weak interaction with the Rab10 $T^{23}N$ mutant, suggesting that the interaction between Rim1 RBD and Rab10 depends on the GTP-binding status of the small GTPase.

To test the pull-down assay, the Rim1 RBD (amino acid 11-399) was subcloned into a GST construct and the fusion protein was purified and conjugated to glutathione-coupled beads as described in Materials and Methods. The purified protein was eluted and subjected to Coomassie blue staining to detect the efficiency of purification (**Figure 2.2D**). To test whether GST-Rim1 RBD can differentially pull down Rab10 in different nucleotide-bound forms, COS-1 cell lysates overexpressing wild-type Rab10 were loaded with GDP or the nonhydrolyzable GTP analog GTPγS and were incubated with GST-Rim1 RBD or GST alone. As indicated in **Figure 2.2E** and **2.2F**, GST-Rim1 RBD but not GST alone pulled-down ten times more of the GTP-bound Rab10 than did the GDP-bound Rab10, indicating that the assay using GST-Rim1 can differentiate between the nucleotide-binding states of Rab10.

To further validate the pull down assay, we tested whether GST-Rim1 RBD can be used to evaluate cellular Rab10 activity. COS-1 cells were transfected with wild type, $Q^{68}L$, or $T^{23}N$ Rab10, and cell lysates were subjected to the GST-Rim1 RBD pull-down assay. GST-Rim1 RBD isolated four times more of the constitutively active $Q^{68}L$ mutant, and 70% less of the inactive $T^{23}N$ mutant than the wild-type Rab10 from the whole cell lysates (**Figure 2.2G and H**). These data, taken together, validated the effectiveness of the GST-Rim1 RBD pull-down assay in evaluating the activation status of Rab10 *in vivo*.

AS160 negatively regulates Rab10 activity *in vivo*

The co-precipitation of AS160 with the Rab10-GDP/AIFx affinity matrix led us to test whether AS160 can catalytically inactivate Rab10 *in vivo*. As shown in **Figure 2.3A and B**, less than 40% of Rab10 was isolated by GST-Rim1 RBD in the presence of overexpressed AS160, compared to the level of activity observed its absence, indicating that AS160 can inactivate Rab10 in these cells. Interestingly, AS160 also decreased the activity of the Rab10 Q68L mutant by 40%, suggesting that overexpressed AS160 may able to hydrolyze the GTP in the Rab10 Q68L mutant. (**Figure 2.3A and C**). Although a mutation of the glutamine to leucine in the catalytic center of small GTPases is able to abolish the intrinsic GTP hydrolysis activity, and has thus been widely used to generate constitutively active GTPases, this glutamine to leucine mutation has a variable effect on GAP-catalyzed GTP hydrolysis [33, 34]. Consistent with this notion, our data suggest that the Q68L mutation in Rab10 does not completely abolish GTP hydrolysis catalyzed by AS160 (**Figure 2.3C**). This may at least partially explain the fact that overexpression of Rab10 Q68L had only a modest stimulatory effect on Glut4 translocation in 3T3L1 adipocytes [21], considering a significant portion of Rab10 Q68L could be converted into the inactive GDP-bound form by AS160 *in vivo*. Due to the substantial effect of AS160 on the Rab10 Q68L mutant, we used this mutant in all subsequent experiments to increase the basal Rab10 activity to an easily detectable level.

To test whether the inhibition of AS160 on Rab10 is mediated by the GAP activity, the effect of the AS160 R973K mutant on Rab10 activity was examined. The arginine finger in the GAP domain has been reported to be essential for the catalytic activity of GAP proteins [35], and substitution of this residue to lysine in AS160 can abolish its effect on
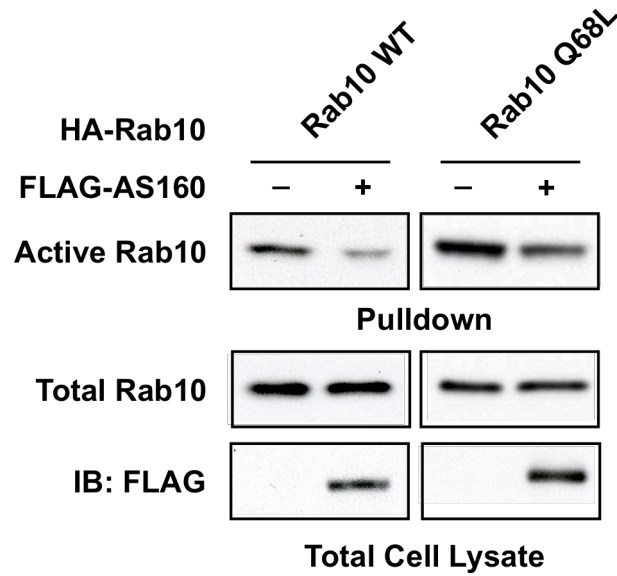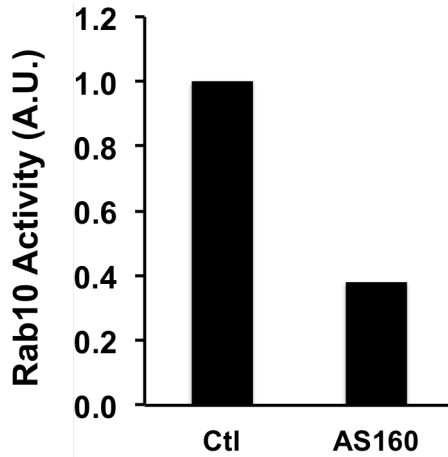
Figure 2.3: AS160 negatively regulates Rab10 activity *in vivo*. A) COS-1 cells were transiently transfected with HA-Rab10 WT or Q68L alone or with FLAG-AS160 constructs as indicated. Cell lysates were incubated with immobilized GST-Rim1 RBD. Pull-downs were resolved on SDS-PAGE and determined by Western Blot with anti-HA antibody. Lysates represent 2% of total protein from a 10-cm plate of COS-1 cells. B) Quantification of Rab10 WT activity by normalizing the pull-down with the total cell lysate. The value of Rab10 activity in the absence of AS160 was then set as one arbitrary unit. C) Quantification of Rab10 Q68L activity from five independent experiments. Significance was determined by Student's *t* test.
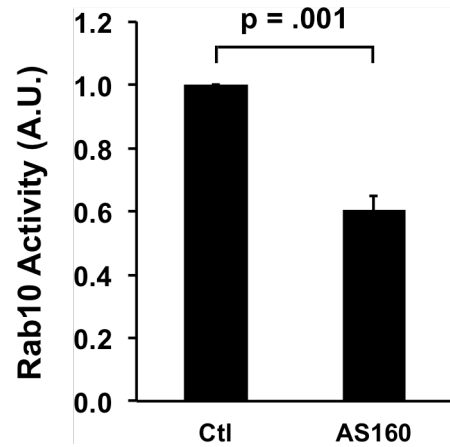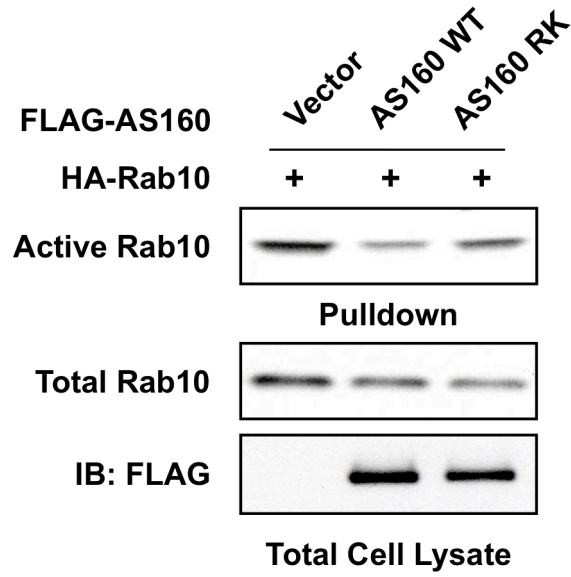
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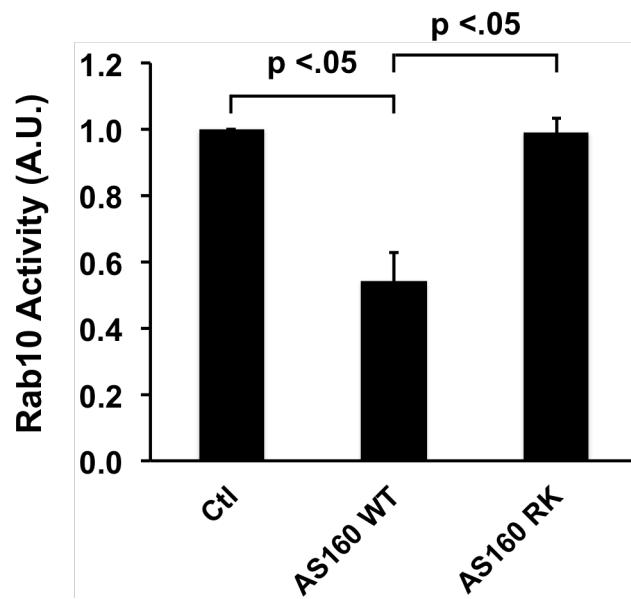
Figure 2.3: AS160 negatively regulates Rab10 activity *in vivo* (continued).
D) COS-1 cells were transiently transfected with HA-Rab10 alone or with
FLAG-AS160 WT or AS160 R973K constructs as indicated. Cell lysates were
incubated with immobilized GST-Rim1 RBD. Pull-downs were subjected to
SDS-PAGE and WB with anti-HA antibody. Lysates represent 2% of total
protein from a 10-cm plate of COS-1 cells. E) Quantification of Rab10 activity
from three independent experiments as described before. Significance was
determined by Student's *t* test.

F
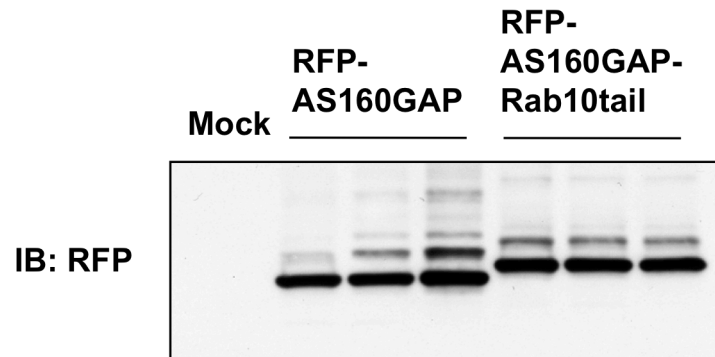
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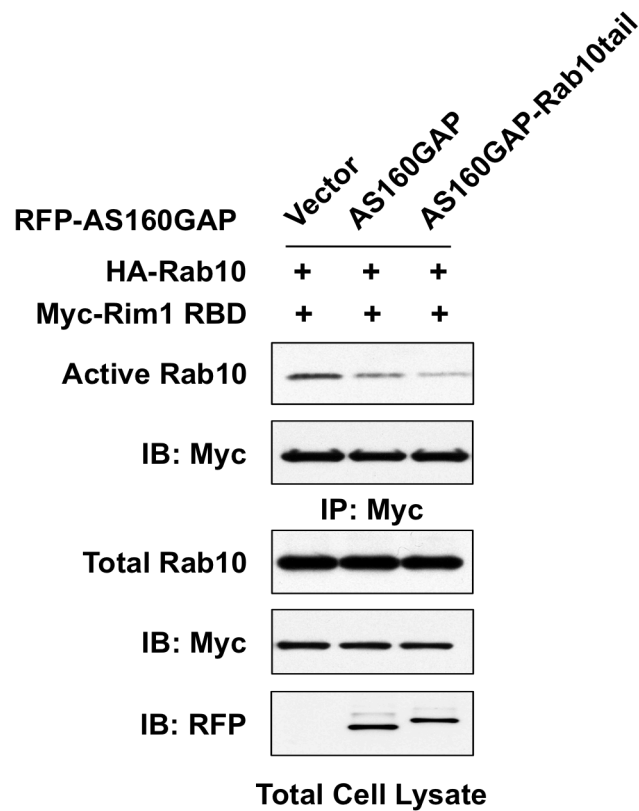
Figure 2.3: AS160 negatively regulates Rab10 activity *in vivo* (continued). F) Western blot analysis of RFP-AS160GAP and RFP-AS160GAP-Rab10tail expression in COS-1 cells. Cells were transiently transfected construct as indicated and lysates were subjected to anti-RFP immunoblotting. G) COS-1 cells were transiently transfected with Myc-Rim1, HA-Rab10 and RFP vector, RFP-AS160GAP or RFP-AS160GAP-Rab10tail constructs as indicated. Whole cell lysates (lower panels) or anti-Myc immunoprecipitates were subjected to immunoblotting with anti-Myc, anti-HA, or anti-RFP antibodies.

Glut4 translocation, potentially due to loss of function of the GAP domain [17]. Indeed, in the GST-Rim1 RBD pull-down assay, while the wild-type AS160 consistently reduced Rab10 activity by 40%, the R973K mutation in the GAP domain completely reversed this inhibition (**Figure 2.3D and E**), indicating that AS160 inactivates Rab10 *in vivo* via GAP-dependent catalysis.

After inactivation by the GAP, small GTPases no longer interact with their effectors, thus switching off the downstream cellular processes. Thus, the association between Rab10 and its downstream effectors is expected to be disrupted by AS160. In this regard, the interaction between Rab10 and its effector Rim1 was largely reduced in the presence of overexpressed AS160-GAP domain (**Figure 2.3G**). To explore whether this effect was due to direct inactivation of Rab10 by AS160 GAP activity, we generated a chimeric protein (AS160GAP-Rab10tail) targeted specifically to Rab10-containing intracellular compartments by attaching the C-terminal sequence (amino acid 168-200) of Rab10 to the GAP domain of AS160 (**Figure 2.3F**). The C-terminal regions of small GTPases are higly variable and undergo post-translational lipid modifications, which serves as targeting signal to localize each small GTPase to a specific membrane [11]. We previously demostrated that the C-terminal 22 residues of H-Ras are sufficient to target the cytosolic protein Gapex-5 to the plasma membrane, where Ras is localized [26]. Intriguingly, the AS160GAP-Rab10tail chimera exhibited stronger inhibition of the interaction of Rab10 with Rim1 than did AS160-GAP alone (**Figure 2.3G**), potentially due to more pronounced targeting of the GAP to pools of Rab10.

Akt phosphorylation negatively regulates AS160 GAP activity

Previous studies have suggested that an intact and functional GAP domain of AS160 is required for blocking insulin-stimulated Glut4 translocation, and that this inhibitory effect may be reduced by Akt-catalyzed phosphorylation [17, 36, 37]. However, currently there is no direct evidence that phosphorylation of AS160 by Akt impacts the regulation of small GTPase targets. The identification of Rab10 as a *bona fide* AS160 target prompted us to investigate whether AS160 might be regulated by Akt-dependent phosphorylation. We first examined the *in vivo* GAP activity of the non-phosphorylable AS160 4A mutant, in which four (Ser^{318}, Ser^{588}, Thr^{642}, Ser^{751}) of the five Akt phosphorylation sites are mutated to alanine [17]. AS160 phosphorylation was readily detectable with the phosphor-Thr^{642} antibody when expressed in COS-1 cells, reflective of the high endogenous Akt activity in these cells (**Figure 2.4A and B**). Intriguingly, AS160-4A showed no phosphorylation, while producing significantly more inhibition of Rab10 activity than seen with expression of wild-type AS160. Collectively, these data indicate that the GAP activity of AS160 towards Rab10 is negatively associated with its phosphorylation status.

To further test this hypothesis, the GAP activity of AS160 was tested in the presence of a myristoylated, constitutively active Akt (Myr-Akt) [16]. AS160 phosphorylation was enhanced in COS-1 cells expressing Myr-Akt comparing to control cells (**Figure 2.4C and D**). Moreover, the inhibitory effect of AS160 on *in vivo* Rab10 activity was almost completely abolished by Myr-Akt phosphorylation. Conversely, Myr-Akt expression did

A

FLAG-AS160	Vector	AS160 WT	AS160 4A
HA-Rab10	+	+	+

Active Rab10

Pulldown

Total Rab10

IB: FLAG

IB: pAS160

Total Cell Lysate
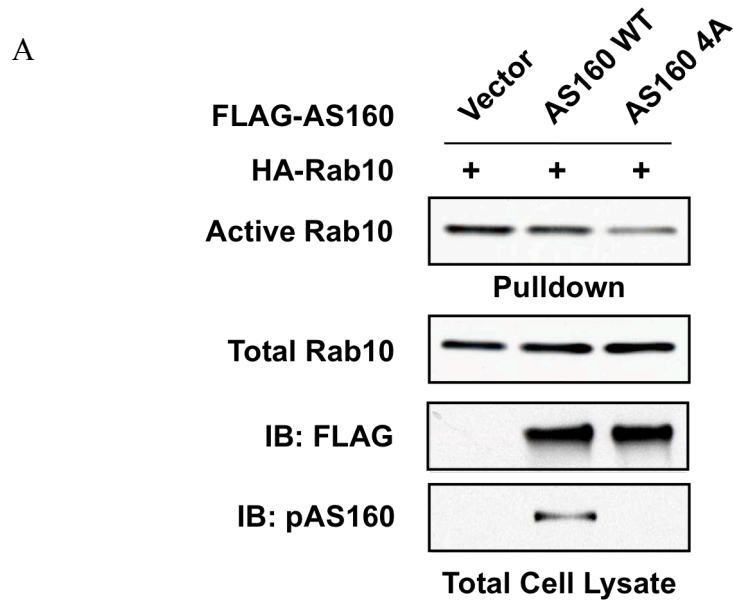
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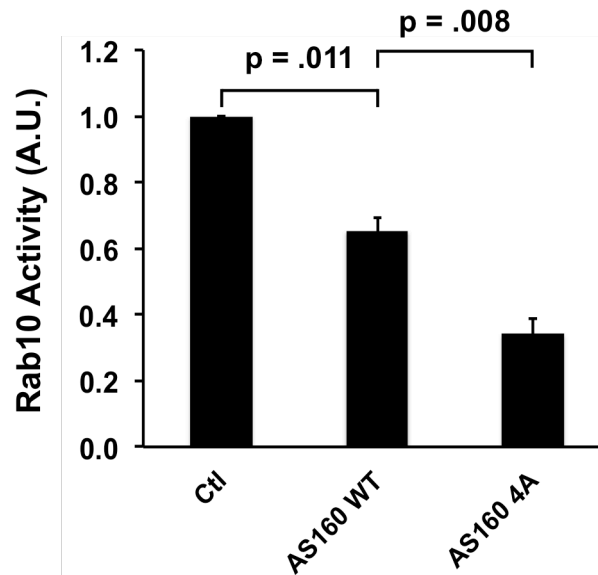
Figure 2.4: Akt phosphorylation negatively regulates AS160 GAP activity towards Rab10. A) COS-1 cells were transiently transfected with HA-Rab10 alone or with FLAG-AS160 WT or AS160 4A constructs as indicated. Cell lysates were incubated with immobilized GST-Rim1 RBD. Associate proteins were separated by SDS-PAGE and detected by Western Blot with anti-HA antibody. Lysates represent 2% of total protein from a 10-cm plate of COS-1 cells. Phosphorylation of AS160 was determined by anti-phosphor-Thr[642] antibody. B) Quantification of Rab10 activity from three independent experiments as described before. Significance was determined by Student's *t* test.

C

	Myr-Akt	−	−	+
	FLAG-AS160 WT	−	+	+
	HA-Rab10	+	+	+

Active Rab10

Pulldown

Total Rab10

IB: FLAG

IB: pAS160

IB: pAkt

Total Cell Lysate

D
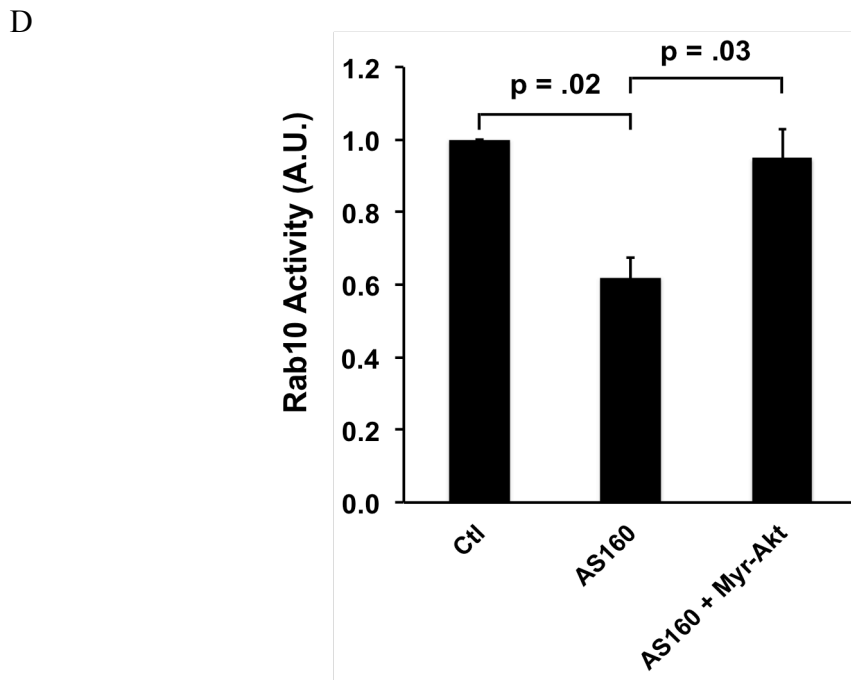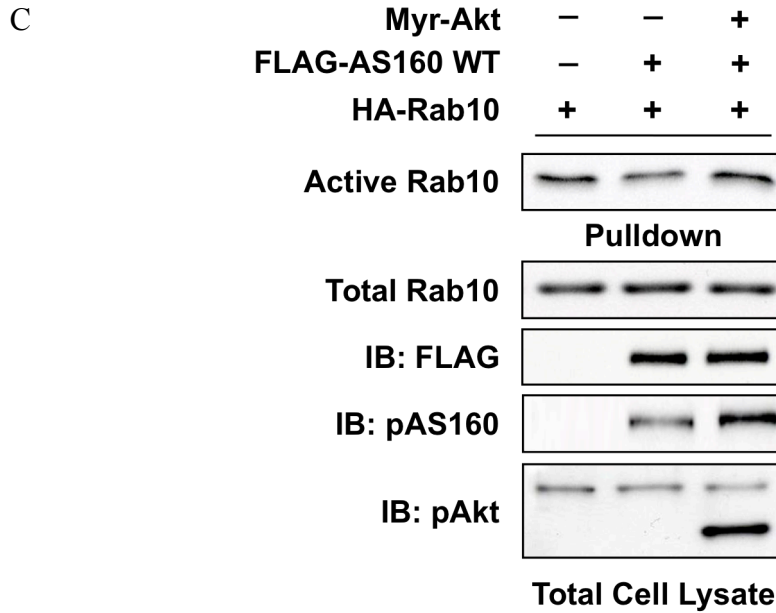
Figure 2.4: Akt phosphorylation negatively regulates AS160 GAP activity towards Rab10 (continued). C) COS-1 cells were transiently transfected with HA-Rab10, with FLAG-AS160 WT or Myr-Akt constructs as indicated. Cell lysates were incubated with immobilized GST-Rim1 RBD. Pull-downs were analyzed by SDS-PAGE and Western Blot with anti-HA antibody. Lysates represent 2% of total protein from a 10-cm plate of COS-1 cells. Phosphorylation of AS160 was determined by anti-phosphor-Thr[642] antibody. Phosphorylation of Akt was determined by anti-phosphor-Thr[308]. D) Quantification of Rab10 activity from three independent experiments as described before. Significance was determined by Student's *t* test.

E

Myr-Akt	−	−	+
FLAG-AS160 4A	−	+	+
HA-Rab10	+	+	+

Active Rab10

Pulldown

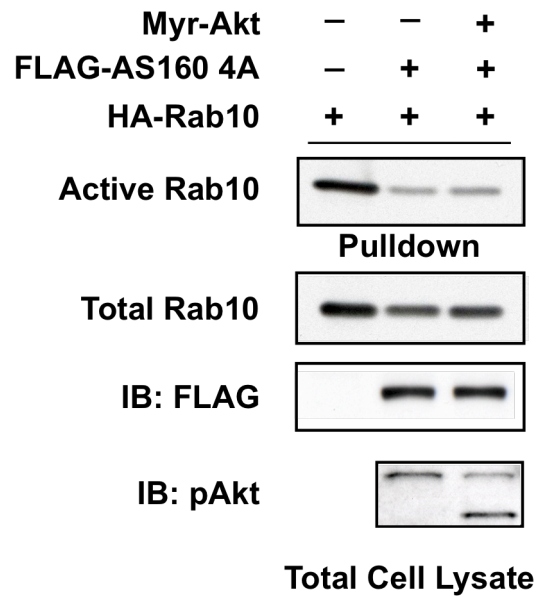Total Rab10

IB: FLAG

IB: pAkt

Total Cell Lysate

Figure 2.4: Akt phosphorylation negatively regulates AS160 GAP activity towards Rab10 (continued). E) COS-1 cells were transiently transfected with HA-Rab10, with FLAG-AS160 4A or Myr-Akt constructs as indicated. Cell lysates were incubated with immobilized GST-Rim1 RBD. Pull-downs were subjected to SDS-PAGE and Western Blot with anti-HA antibody. Lysates represent 2% of total protein from a 10-cm plate of COS-1 cells. Phosphorylation of Akt was determined by anti-phosphor-Thr[308].

not produce any detectable phosphorylation of AS1604A, nor did it influence the GAP

activity of the non-phosphorylable mutant (**Figure 2.4E**). Taken together, these data

provide the first direct evidence that phosphorylation of AS160 by Akt negatively

regulates AS160 GAP activity towards its substrate.

Discussion

The data presented here identifies Rab10 as a functional small GTPase target of the GAP

protein AS160. We also directly demonstrate that the GAP activity of AS160 towards

Rab10 is negatively regulated by Akt-catalyzed phosphorylation. AS160 was found to be

phosphorylated in response to insulin on five Akt consensus sites [17]. Mutation of four

of these phosphorylation sites into non-phosphorylable alanine blocked Glut4

translocation [17, 23, 38], suggesting that Akt phosphorylation may negatively regulate

AS160 activity. Our results demonstrating that the GAP activity of AS160 against its

target Rab10 is inhibited by Akt-induced phosphorylation provides the first direct

evidence supporting this hypothesis.

These data suggest two mechanisms by which AS160 may be regulated by Akt

phosphorylation: 1) phosphorylation by Akt may directly inhibit the enzymatic activity of

AS160, resulting in reduced Rab10 GTP hydrolysis; or 2) Akt phosphorylation may lead

to a spatial relocation of AS160, sequestering it from its substrate small GTPase. The fact

that none of the Akt phosphorylation sites is located within its GAP domain may argue

against the role for phosphorylation in regulation of the catalytic activity of AS160.

However, it is still possible that the phosphorylation of AS160 could result in a conformational change that may decrease its affinity to Rab10. In addition, recent studies reported that phosphorylation on Thr^{642} of AS160 by Akt created a binding site for the 14-3-3 family members, which negatively regulate AS160 activity, potentially by decreasing its accessibility to downstream targets [6, 39].

Materials and Methods

Antibodies

The anti-HA and anti-myc monoclonal antibodies were purchased from Santa Cruz Biotechnology. The anti-FLAG M2 monoclonal antibody was purchased from Sigma-Aldrich. The anti-RFP polyclonal antibody was purchased from Abcam. The anti-phospho-Akt (Thr308) polyclonal antibody was purchased from Cell Signaling Technology. The anti-phospho-AS160 (Thr642) was from Millipore.

Expression Constructs

The N-terminal Rab binding domain (RBD) of Rim1 (residues 11-399) was cloned from Marathon-Ready adult brain cDNA (Clontech) by PCR and subcloned into pKCmyc (myc tag) vector and pGEX (GST tag) vector as described in chapter 2. Rab4, 5, 10 and 11 and RalA cDNA were from lab stock and subcloned into pKH3 (HA tag) vector or pGEX vector. AS160 cDNA was from Dr. Lienhard and subcloned into p3XFLAG-CMV

(FLAG tag). Point mutants of Rab10 and AS160 were created using the QuikChange method (Stratagene). The Myr-Akt construct was kindly provided by A. Vojteck (University of Michigan). To make the AS160GAP-Rab10tail chimera, the GAP domain of AS160 was amplified by PCR with a 3' primer containing the last 132 bp of Rab10 cDNA, and subcloned into the RFP vector, which was kindly provided by Dr. Roger Tsien (UCSD). Automatic DNA sequencing was performed at the University of Michigan DNA Sequencing Core (Ann Arbor, MI). All constructs were sequenced to confirm reading frame.

Preparation of GST-fusion proteins

GST alone or GST-RalA, Rab4, 5, 10 and 11 were prepared similarly as described in chapter 2. Briefly, BL21(DE3)pLysS bacteria was transformed with Plasmids pGEX-2T expressing Glutathione S-Transferase (GST) alone or the indicated GST-fusion protein, and then inoculated into 500 ml LB containing 100 µg/ml ampicillin. When bacteria concentration reached an OD_{600}=0.7, protein expression was induced with IPTG added to a final 0.1 mM concentration, and with the bacteria incubated at $16\,^{o}C$ for 20 hours with constant agitation of 250 rpm. The cells were pelleted at 5,000xg for 10 minutes then resuspended in ice-cold 20 ml TEN (50 mM Tris, pH 7.5, 0.5 mM EDTA, 0.3 M NaCl) with 200 µl 100 mg/ml lysozyme added to break down the cell wall. After 15 minutes incubation on ice, 400 µl 10% NP-40 was added for 10 minutes to solve the plasma membrane. The mixture was snap-frozen in liquid nitrogen, and then allowed to thaw slowly at room temperature. Two Complete Mini Protease inhibitor cocktail tablets

(Roche Diagnostics) were dissolved in 30 ml ice-cold NaCl-Mg (1.5M NaCl, 12 mM MgCl$_2$) and then added to cell lysate. 1ml 10 mg/ml DNase I was then added. The lysate was then centrifuged for 20 minutes at 15,000xg, and the supernatant was stored in 1 mL aliquots at -70°C.

To couple GST or GST-fusion proteins to glutathione-coupled beads (GE healthcare), 1ml lysates was incubated with 200 μl 1:1 pre-washed glutathione-beads in PBS (Phosphate Buffered Saline, pH 7.4) at 4°C for 2 hr with constant rotation. The glutathione-beads bound GST fusion proteins were washed three times with 1 ml PBS, pH 7.4, and re-suspended in 200 μl 50% Glycerol in PBS, pH 7.4. To determine protein concentrations, aliquots of GST or GST-fusion proteins bound to glutathione agarose were diluted with 5 μl 2X SDS-PAGE sample buffer, and analyzed by SDS-PAGE and Coomassie blue staining.

Cell culture and transfections

COS-1 cells were maintained in DMEM containing 25mM glucose, 10% fetal bovine serum at 37°C with 5% CO$_2$. COS-1 cells were transfected with DNA using FuGENE6 transfection reagent (Roche, Indianapolis, IN).

GDP-AIFx loading assay

GST fusion proteins were expressed and purified as previously described. GST-Rab10 bound to glutathione beads was incubated for 30 min at 25°C in loading buffer (20 mM Tris, pH 7.5, 1 mM dithiothreitol (DTT), 50 mM NaCl) with 2 mM EDTA and Complete, EDTA-free protease inhibitor tablets. GST-Rab10 was then loaded with nucleotide by incubating in loading buffer supplemented with 2 mM GDP or 200 mM GTPγS for 1 h at 25°C. To stop loading, 10 mM $MgCl_2$ was added for 5 min at 25°C. To induce GDP/AlFx-bound Rab10, 30 mM $AlCl_3$ and 10 mM NaF were included in the GDP loading buffer. Loaded GST-Rab10 beads were then added to COS-1 cells that were lysed in buffer (25 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol, 1% NP-40, 5 mM $MgCl_2$, and 1 mM DTT) supplemented with 10 μM GDP; 10 μM GTPγS; or 10 μM GDP, 30 μM $AlCl_3$, and 10 mM NaF, and Complete, EDTA-free protease inhibitor tablets. The reaction was incubated for 2 h at 4°C, and then beads were washed three times in wash buffer (25 mM Tris, pH 7.5, 40 mM NaCl, 30 mM $MgCl_2$, 1% NP-40, and 1 mM DTT) and once in rinse buffer (25 mM Tris, pH 7.5, 40 mM NaCl, 30 mM $MgCl_2$, and 1 mM DTT) supplemented with 10 μM GDP; 10 μM GTPγS; or 10 μM GDP, 30 mM $AlCl_3$, and 10 mM NaF, and Complete EDTA-free protease inhibitor tablets. The pull-downs were solubilized in 2X SDS sample buffer and subjected to SDS–PAGE and western blotting was performed using the indicated antibodies.

GST-Rim1 pull-down assay for *in vivo* Rab10 activity

COS-1 cells were lysed with lysis buffer (25 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol, 1% NP-40, 5 mM $MgCl_2$, and 1 mM DTT) supplemented with Complete,

EDTA-free protease inhibitor tablets and with 10 μM GDP; or 10 μM GTPγS if indicated.

Cell lysates were then incubated with either GST alone or GST-Rim1 RBD bound to

glutathione-Sepharose beads (GE healthcare) for 35 min at 4°C. The beads were washed

four times with lysis buffer, re-suspended in 2X sample buffer, subjected to SDS-PAGE,

and analyzed by immunoblotting. Amounts of Rab10 pulled down by GST-Rim1 were

quantified with Image J and compared with amounts of Rab10 in the total cell lysates to

determine the proportions of Rab10 activation.

Immunoprecipitation and immunoblotting

Cell were washed three times with ice-cold PBS (phosphate-buffered saline) and then

lysed on ice for 10 min with 1 ml lysis buffer (100 mM Tris, pH 7.5, 1% NP-40, 130 mM

NaCl, 1 mM Na$_3$VO$_4$, 5 mM MgCl$_2$, 1 mM EDTA, and 10 mM NaF) with Complete,

EDTA-free protease inhibitor tablets (Roche). Whole cell lysates were centrifugated for

10 min at 13,000 × g. Lysates were incubated with 4 μg of the indicated antibody for 2 h

at 4°C, and then protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) were

added for 2 h at 4°C to precipitate the antibody. Beads were washed three times in lysis

buffer and then resuspended in 2X SDS sample buffer. The immunoprecipitates were

subjected to SDS-PAGE and western blotting was performed using the indicated

antibodies.

References

1. Saltiel, A.R. and C.R. Kahn, *Insulin signalling and the regulation of glucose and lipid metabolism.* Nature, 2001. **414**(6865): p. 799-806.

2. Watson, R.T., M. Kanzaki, and J.E. Pessin, *Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes.* Endocr Rev, 2004. **25**(2): p. 177-204.

3. Bryant, N.J., R. Govers, and D.E. James, *Regulated transport of the glucose transporter GLUT4.* Nat Rev Mol Cell Biol, 2002. **3**(4): p. 267-77.

4. Hou, J.C. and J.E. Pessin, *Ins (endocytosis) and outs (exocytosis) of GLUT4 trafficking.* Curr Opin Cell Biol, 2007. **19**(4): p. 466-73.

5. Watson, R.T. and J.E. Pessin, *Bridging the GAP between insulin signaling and GLUT4 translocation.* Trends Biochem Sci, 2006. **31**(4): p. 215-22.

6. Sakamoto, K. and G.D. Holman, *Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic.* Am J Physiol Endocrinol Metab, 2008. **295**(1): p. E29-37.

7. Whiteman, E.L., H. Cho, and M.J. Birnbaum, *Role of Akt/protein kinase B in metabolism.* Trends Endocrinol Metab, 2002. **13**(10): p. 444-51.

8. Ishikura, S., A. Koshkina, and A. Klip, *Small G proteins in insulin action: Rab and Rho families at the crossroads of signal transduction and GLUT4 vesicle traffic.* Acta Physiol (Oxf), 2008. **192**(1): p. 61-74.

9. Welsh, G.I., et al., *Regulation of small GTP-binding proteins by insulin.* Biochem Soc Trans, 2006. **34**(Pt 2): p. 209-12.

10. Grosshans, B.L., D. Ortiz, and P. Novick, *Rabs and their effectors: achieving specificity in membrane traffic.* Proc Natl Acad Sci U S A, 2006. **103**(32): p. 11821-7.

11. Takai, Y., T. Sasaki, and T. Matozaki, *Small GTP-binding proteins.* Physiol Rev, 2001. **81**(1): p. 153-208.

12. Cai, H., K. Reinisch, and S. Ferro-Novick, *Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle.* Dev Cell, 2007. **12**(5): p. 671-82.

13.   Hall, A., *Rho GTPases and the actin cytoskeleton.* Science, 1998. **279**(5350): p. 509-14.

14.   Bos, J.L., H. Rehmann, and A. Wittinghofer, *GEFs and GAPs: critical elements in the control of small G proteins.* Cell, 2007. **129**(5): p. 865-77.

15.   Chen, X.W., et al., *Activation of RalA is required for insulin-stimulated Glut4 trafficking to the plasma membrane via the exocyst and the motor protein Myo1c.* Dev Cell, 2007. **13**(3): p. 391-404.

16.   Chen, X.W., et al., *A Ral GAP complex links PI 3-kinase/Akt signaling to RalA activation in insulin action.* Mol Biol Cell, 2011. **22**(1): p. 141-52.

17.   Sano, H., et al., *Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation.* J Biol Chem, 2003. **278**(17): p. 14599-602.

18.   Thong, F.S., P.J. Bilan, and A. Klip, *The Rab GTPase-activating protein AS160 integrates Akt, protein kinase C, and AMP-activated protein kinase signals regulating GLUT4 traffic.* Diabetes, 2007. **56**(2): p. 414-23.

19.   Kane, S., et al., *A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain.* J Biol Chem, 2002. **277**(25): p. 22115-8.

20.   Miinea, C.P., et al., *AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain.* Biochem J, 2005. **391**(Pt 1): p. 87-93.

21.   Sano, H., et al., *Rab10, a target of the AS160 Rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane.* Cell Metab, 2007. **5**(4): p. 293-303.

22.   Sano, H., et al., *Rab10 in insulin-stimulated GLUT4 translocation.* Biochem J, 2008. **411**(1): p. 89-95.

23.   Ishikura, S., P.J. Bilan, and A. Klip, *Rabs 8A and 14 are targets of the insulin-regulated Rab-GAP AS160 regulating GLUT4 traffic in muscle cells.* Biochem Biophys Res Commun, 2007. **353**(4): p. 1074-9.

24.   Scheffzek, K., et al., *The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants.* Science, 1997. **277**(5324): p. 333-8.

25.   Wittinghofer, A., *Signaling mechanistics: aluminum fluoride for molecule of the year.* Curr Biol, 1997. **7**(11): p. R682-5.

26.   Lodhi, I.J., et al., *Gapex-5, a Rab31 guanine nucleotide exchange factor that regulates Glut4 trafficking in adipocytes.* Cell Metab, 2007. **5**(1): p. 59-72.

27.   Fukuda, M., *Distinct Rab binding specificity of Rim1, Rim2, rabphilin, and Noc2. Identification of a critical determinant of Rab3A/Rab27A recognition by Rim2.* J Biol Chem, 2003. **278**(17): p. 15373-80.

28.   Babbey, C.M., et al., *Rab10 regulates membrane transport through early endosomes of polarized Madin-Darby canine kidney cells.* Mol Biol Cell, 2006. **17**(7): p. 3156-75.

29.   Stenmark, H., et al., *Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis.* EMBO J, 1994. **13**(6): p. 1287-96.

30.   Bucci, C., et al., *Rab7: a key to lysosome biogenesis.* Mol Biol Cell, 2000. **11**(2): p. 467-80.

31.   Peranen, J., et al., *Rab8 promotes polarized membrane transport through reorganization of actin and microtubules in fibroblasts.* J Cell Biol, 1996. **135**(1): p. 153-67.

32.   Wang, E., et al., *Apical and basolateral endocytic pathways of MDCK cells meet in acidic common endosomes distinct from a nearly-neutral apical recycling endosome.* Traffic, 2000. **1**(6): p. 480-93.

33.   Pan, X., et al., *TBC-domain GAPs for Rab GTPases accelerate GTP hydrolysis by a dual-finger mechanism.* Nature, 2006. **442**(7100): p. 303-6.

34.   De Antoni, A., et al., *Significance of GTP hydrolysis in Ypt1p-regulated endoplasmic reticulum to Golgi transport revealed by the analysis of two novel Ypt1-GAPs.* J Biol Chem, 2002. **277**(43): p. 41023-31.

35.   Ahmadian, M.R., et al., *Confirmation of the arginine-finger hypothesis for the GAP-stimulated GTP-hydrolysis reaction of Ras.* Nat Struct Biol, 1997. **4**(9): p. 686-9.

36.   Eguez, L., et al., *Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein.* Cell Metab, 2005. **2**(4): p. 263-72.

37.   Larance, M., et al., *Characterization of the role of the Rab GTPase-activating protein AS160 in insulin-regulated GLUT4 trafficking.* J Biol Chem, 2005. **280**(45): p. 37803-13.

38.   Sun, Y., et al., *Rab8A and Rab13 are activated by insulin and regulate GLUT4 translocation in muscle cells.* Proc Natl Acad Sci U S A, 2010. **107**(46): p. 19909-14.

39.   Ramm, G., et al., *A role for 14-3-3 in insulin-stimulated GLUT4 translocation through its interaction with the RabGAP AS160.* J Biol Chem, 2006. **281**(39): p. 29174-80.

Chapter 3

Rab10 and RalA participate in a GTPase cascade

Introduction

Insulin increases glucose transport into fat and muscle cells by promoting translocation of the facilitative glucose transporter Glut4 from intracellular sites to the plasma membrane [1-3]. In the basal state, Glu4 slowly but constitutively recycles between the cytoplasm and plasma membrane, with a steady state distribution favoring the intracellular compartments [1, 4]. Insulin stimulation causes a 10-fold increase of Glut4 levels in the plasma membrane, primarily by enhancing the exocytosis of Glut4-residing vesicles through regulated translocation, docking and membrane fusion [3, 5]. The precise mechanisms coordinating the signaling events and the transport machineries remaining poorly understood [6, 7].

The small GTPases act as "molecular switches" that alternate between two conformational states—the GTP-bound active state and GDP-bound inactive state. Upon activation, small GTPases can recruit downstream effector proteins that regulate distinct steps of vesicle exocytosis, including vesicle budding, traveling along the cytoskeleton, tethering/docking at the designated site and fusion with the target membrane [7-9]. Members of the Ras, Rho and Rab subfamilies of small GTPases have been reported to regulate multiple steps of Glut4 vesicle trafficking [3, 10-13]. One such example is the Ras subfamily member RalA, which is activated in response to insulin, and regulates

Glut4 exocytosis through recognition of the exocyst complex and the interaction with molecular motor Myo1c [12]. A Rab subfamily GTPase Rab10 has also been suggested to be required for insulin-stimulated Glut4 translocation, although the downstream effectors through which Rab10 regulates this process remain unknown [14, 15].

The activation status of small GTPases is regulated by GEFs (guanine nucleotide exchange factors) that facilitate the exchange of GDP for GTP, and GAPs (GTPase activating proteins) that promote GTP hydrolysis [16]. In many scenarios, the activities of GEFs and GAPs are tightly regulated by upstream signaling [16, 17]. One example of such regulation is the RalA GAP complex RGC1/2 [18, 19], which has several Akt kinase phosphorylation sites and a GAP domain that maintains RalA in a low activity state. In response to insulin, Akt directly phosphorylates RGC proteins, and negatively regulates the GAP activity of the RGC complex to RalA, leading to increased RalA activation and consequently enhanced plasma membrane Glut4 levels and increased glucose uptake [18]. Another example of such regulation is the Rab10 GAP protein AS160, the GAP activity of which has been demonstrated to be negatively regulated by Akt phosphorylation in chapter 2 of this dissertation.

While RGC1/2 is the only characterized RalGAP to date, there are at least six different members of the RalGEF family [20-26], which is subdivided into the RalGDS and RalGPS subfamilies. The RalGDS subfamily members all contain a Ras binding domain (RBD) and are under the regulation of activated Ras GTPases [21, 22, 24, 25, 27]. In contrast, the RalGPS subfamily members lack an RBD and may respond to other

upstream signals such as phosphorylation [20, 23, 26]. In response to extracellular signals, such as receptor tyrosine kinases, Ras is activated and recruits the RalGDS in proximity of membrane compartments where they can access and activate RalA [27-29]. It is still unknown whether RalA is activated by insulin through the Ras-RalGDS pathway, and whether this pathway participates in mediating the insulin-stimulated Glut4 translocation. Here we propose that the Rab GTPase Rab10 regulates RalA activity, possibly through a yet unidentified RalGEF.

Results

Effect of RGC2 and AS160 depletion on glucose uptake

Previous studies have revealed the two Akt substrate proteins RGC2 and AS160 as negative regulators of glucose uptake and Glut4 translocation [18, 30]. To study whether the two proteins synergistically regulate insulin-stimulated glucose uptake, siRNA oligos were applied to knock down both RGC2 and AS160 in 3T3-L1 adipocytes. As shown in **Figure 3.1A**, AS160 siRNA and RGC2 siRNA oligos abolished the expression of both proteins in 3T3-L1 adipocytes without affecting upstream signaling events. Consistent with previous reports, knockdown of either AS160 or RGC2 caused an increase in glucose uptake at the low doses of insulin, confirming the negative roles of the two proteins in regulating Glut4 translocation. Interestingly, however, depletion of both AS160 and RGC2 were not additive compared to depletion of either protein alone (**Figure 3.1B**). One possible explanation for this lack of additive effect is that AS160 and

A
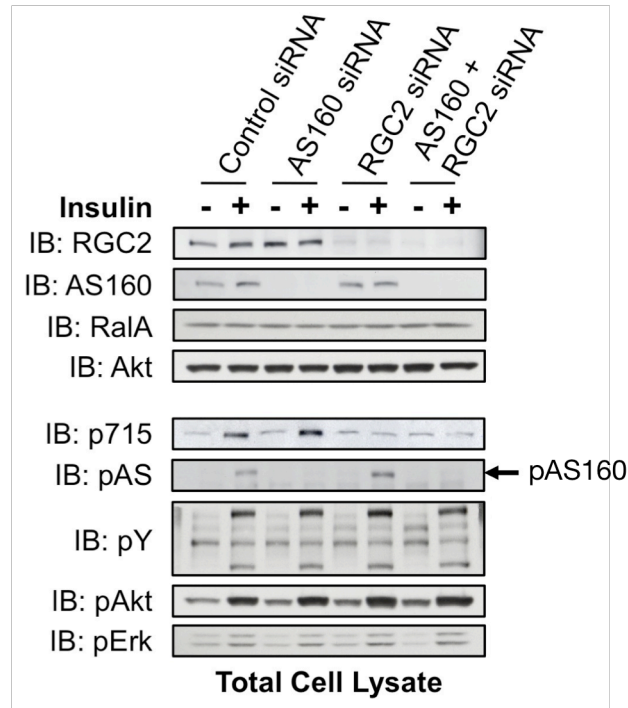
B
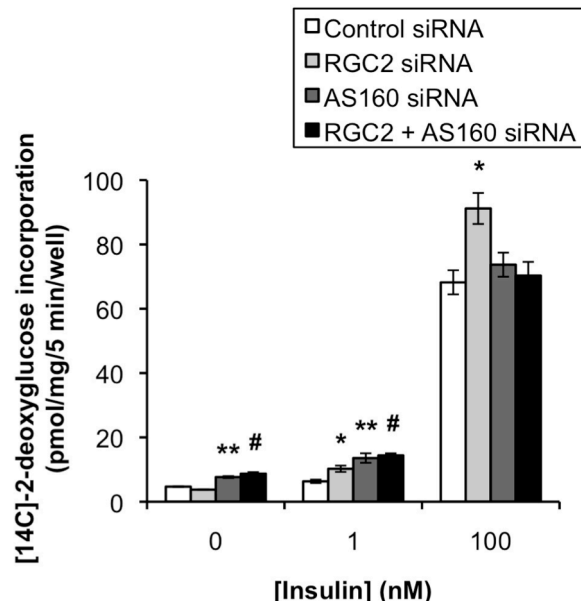
Figure 3.1: Depletion of RGC2 and AS160 has no additive effect on glucose uptake. A) siRNA knockdown of RGC2 and AS160 does not affect insulin signaling pathway in 3T3-L1 adipocytes. B) Insulin-stimulated 2-deoxyglucose uptake by 3T3-L1 adipocytes after transfected with control, RGC2, and/or AS160 siRNA oligos as indicated. **(Courtesy of Dr. Dara Leto, unpublished data)**

111

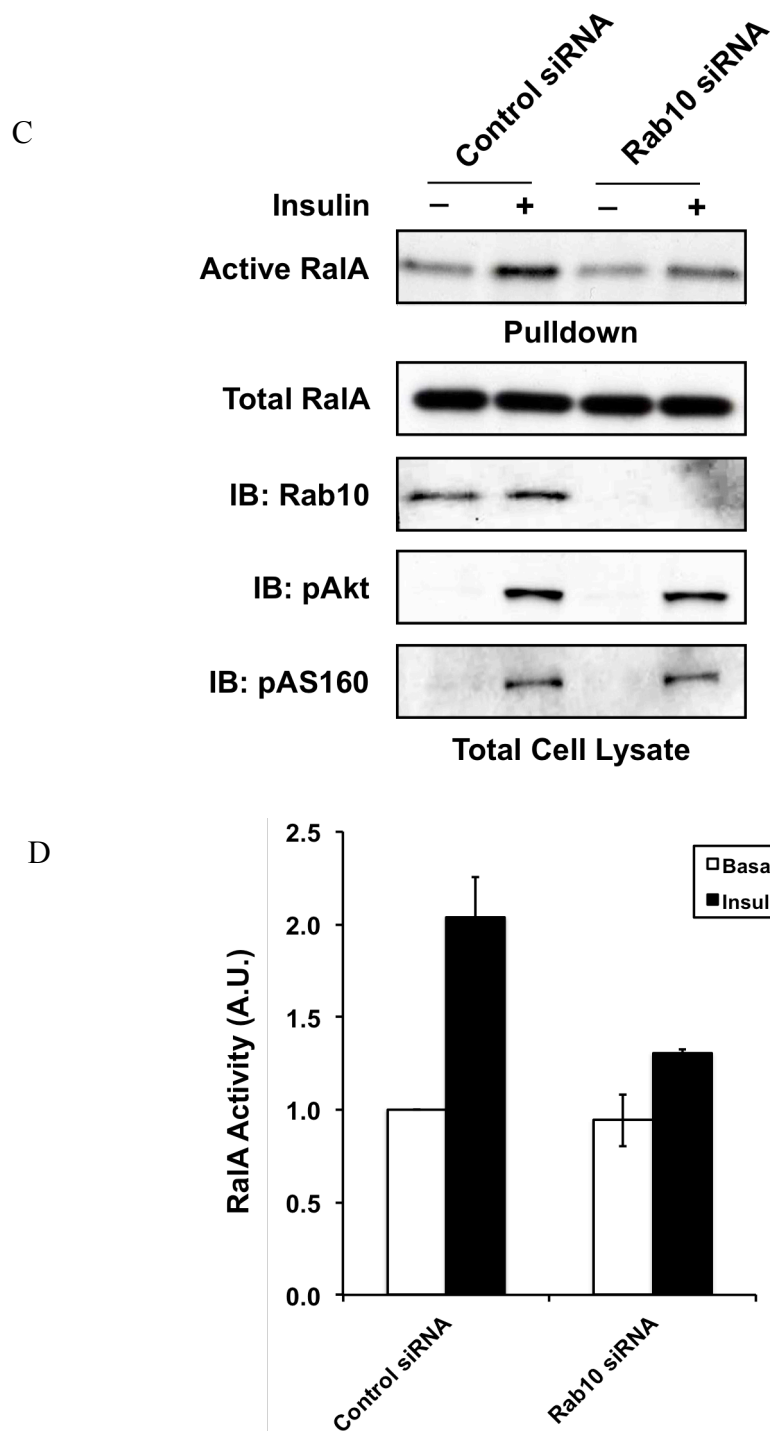RGC2 may have convergent downstream targets in regulating Glut4 translocation, or may reside in a linear pathway.

Rab10 regulates RalA activity

The possibility that the downstream targets of AS160 and RGC2 may converge at some level prompted us to explore the possible crosstalk between Rab10 and RalA, two identified small GTPase substrates of AS160 and RGC2 respectively. RalA interacts with the exocyst complex, at the distal end of Glut4 vesicle trafficking events [12]. In contrast, Rab10 has no identified downstream effectors. We thus examined the effect of Rab10 on RalA activity with the effector pull-down assay described above [12]. Because RalBP1 is a known effector protein of Ral GTPases [31, 32], intracellular RalA activity can be assessed by pull-down with the immobilized RBD of RalBP1. The validity of this assay was further evaluated with the known GEF protein of Ral GTPases Rlf [25, 33]. As indicated in **Figure 3.2A**, Rlf expression resulted in a robust activation of RalA, indicated by increased RalA pulled down by RalBP1 RBD, suggesting that the assay was effective in evaluating RalA activity. Overexpression of the constitutively active Rab10 (Rab10 $Q^{68}L$) resulted in increased RalA activity compared to vector alone, while the inactive Rab10 (Rab10 $T^{23}N$) was without effect (**Figure 3.2A and B**).

The role of Rab10 in regulating RalA activity was further examined by testing whether Rab10 is required for stimulation of RalA activity by insulin. As previously reported,

A

Pulldown

Total RalA

IB: HA

Total Cell Lysate

B
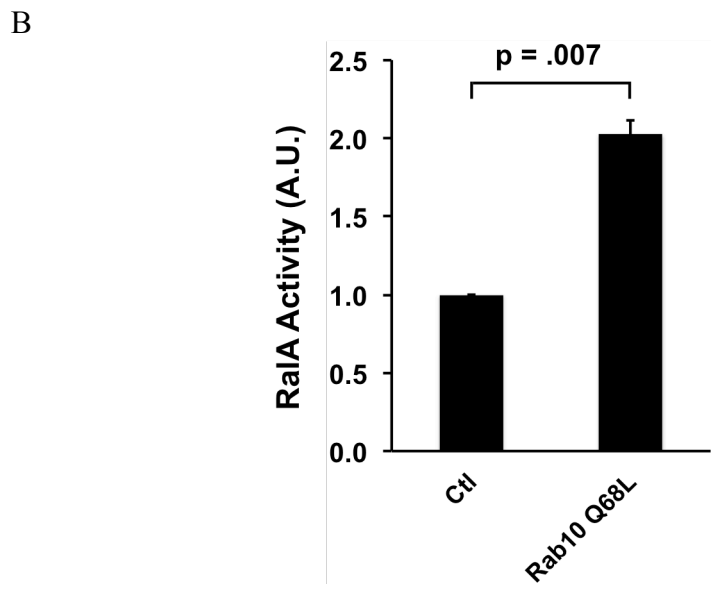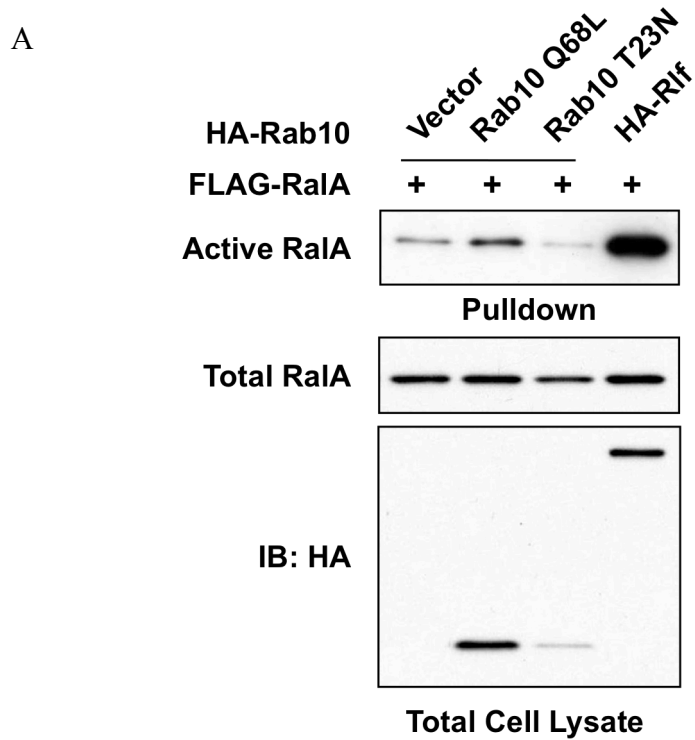
Figure 3.2: Effect of Rab10 on RalA activity. A) COS-1 cells were transiently transfected with FLAG-RalA, and HA-Rab10 Q68L, T23N or HA-Rlf constructs as indicated. Cell lysates were incubated with immobilized RBP1 RBD. Pull-downs were subjected to SDS-PAGE and determined by Western Blot (WB) with anti-FLAG antibody. Lysates represent 2% of total protein from a 10-cm plate of COS-1 cells. B) Quantification of RalA activity from three independent experiments by normalizing the pull-down with the total cell lysate. The value of the activity of RalA in the absence of Rab10 Q68L was then set as one arbitrary unit. Significance was evaluated with Student's *t* test.

C

	Control siRNA		Rab10 siRNA	
Insulin	−	+	−	+

Active RalA

Pulldown

Total RalA

IB: Rab10

IB: pAkt

IB: pAS160

Total Cell Lysate

D

Figure 3.2: Effect of Rab10 on RalA activity (continued). C) 3T3-L1 adipocyte lysates were transfected with control siRNA or siRNA oligos that deplete Rab10. Four days after transfection, cells were mock treated or stimulated with 100 nM insulin for 5 min and lysates were subjected to RBP1 RBD pull-down assay to detect RalA activation. Pull-downs represent the amount of active RalA from half a 10-cm plate of cells. Lysates represent 2% of total protein from a 10-cm plate of cells. D) Quantification of RalA activity upon Rab10 knockdown from three independent experiments as described before. The activity or RalA from control 3T3L-1 adipocytes in the basal state was set as one arbitrary unit.
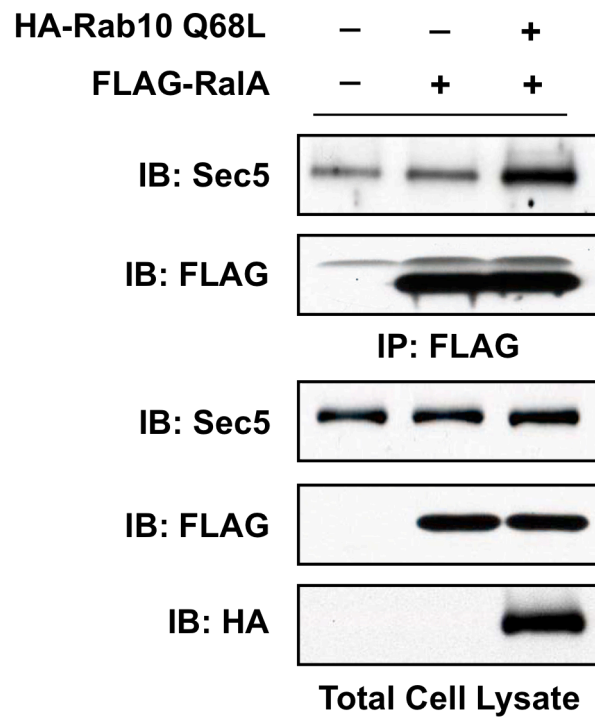
E

Figure 3.2: Effect of Rab10 on RalA activity (continued). E) COS-1 cells were transiently transfected with FLAG-RalA and/or HA-Rab10 Q68L constructs as indicated. Whole cell lysates (lower panels) or anti-FLAG immunoprecipitates were subjected to immunoblotting with anti-FLAG, anti-Sec5, or anti-HA antibodies.

RalA activity in 3T3-L1 adipocytes is stimulated by insulin in a time- and dose-dependent manner [12]. Consistent with previous observations, insulin produced a two-fold activation of RalA in 3T3-L1 adipocytes transfected with scrambled siRNA. Knock down of Rab10 significantly blocked insulin-stimulated RalA activation without affecting the upstream signaling, as indicated by the normal phosphorylation of Akt and the Akt substrate AS160 (**Figure 3.2C and D**). Taken together, these data suggest that Rab10 is both necessary and sufficient for RalA activation.

Previous studies have reported that RalA, together with the exocyst complex, plays a regulatory role in a variety of cellular processes, such as tumorigenesis and insulin-stimulated Glut4 exocytosis [12, 34-36]. RalA has been shown to directly interact with the exocyst subunits Sec5 and Exo84 in a GTP-dependent manner [37, 38]. We thus tested whether Rab10 affects the association of RalA with its effector protein Sec5 by co-immunoprecipitation. RalA interacted with Sec5 in COS-1 cells. Interestingly overexpression of active Rab10 increased this interaction, presumably through increasing the portion of GTP-bound active form of RalA (**Figure 3.2E**).

Mechanism of Rab10 regulation of RalA activity

The regulatory mechanisms of RalA activity have been extensively investigated in previous studies. Similar to other small GTPases, RalA activity is mainly regulated by two groups of special molecules: the RalGAPs and RalGEFs. The RalA GAP complex,

RGC1/2 is phosphorylated by Akt in response to insulin, which inhibits the GAP activity

of RGC1/2 towards RalA [18]. It is therefore possible that Rab10 may activate RalA

through inhibiting RGC1/2 by enhancing the phosphorylation of the complex. To test this

hypothesis, insulin-stimulated RGC phosphorylation in 3T3-L1 adipocytes was examined

after siRNA-mediated knockdown of Rab10. The RGC phosphorylation was determined

with phosphospecific antibodies described in [18], which detected no difference in

control siRNA and Rab10 siRNA oligos treated 3T3-L1 adipocytes (Chen and Saltiel *et

al.*, unpublished data), suggesting that the RalGAP RGC complex may not be implicated

in the activation of RalA by Rab10.

The lack of effect of Rab10 on the GAP activity of RalA led us to test whether Rab10

may regulate RalA activity through a RalGEF. To achieve this goal, we took advantage

of a RalA mutant (RalA $F^{39}L$), which promotes fast cycling between GDP and GTP

bound states, thus mimicking the interaction with RalGEFs [12, 36]. RalA $F^{39}L$ responds

to inhibition by RalGAP [18], rendering it an ideal tool to discriminate between the

involvement of a RalGAP or RalGEF in RalA activation. Theoretically, if Rab10

activates RalA through inhibition of a RalGAP, activation of the RalA $F^{39}L$ mutant by

Rab10 would be expected, similar to what was observed with the wild type RalA.

However, if Rab10 activates RalA through increasing RalGEF activity, no effect of

Rab10 on RalA F39L activity would be expected, as the mutant does not require a

RalGEF for activation. As shown in **Figure 3.3**, the RalBP1 pull-down assay revealed

that while Rab10 expression consistently increased wild type RalA activity, it failed to

activate the RalA F39L mutant, suggesting that Rab10 regulates RalA activity through a
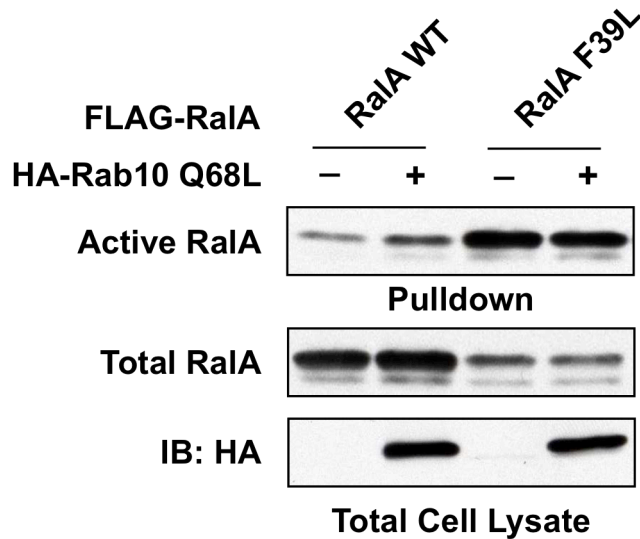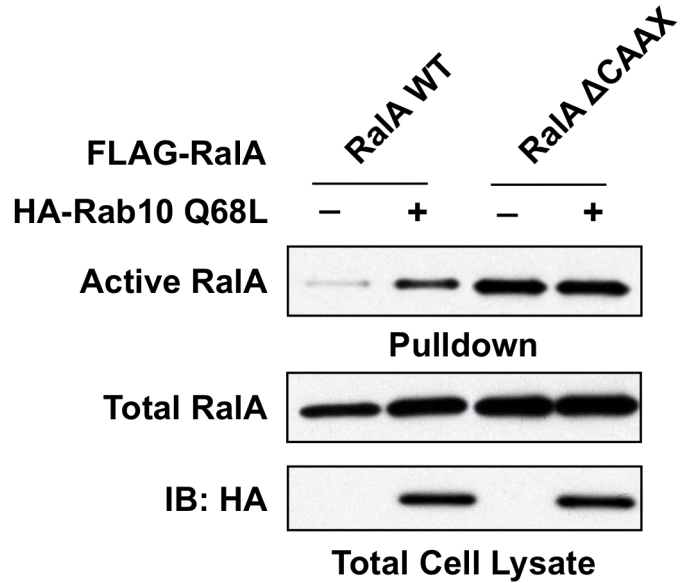
Figure 3.3: Rab10 activates RalA in a RalGEF dependent manner. COS-1 cells were transiently transfected with FLAG-RalA WT, RalA F39L, and HA-Rab10 Q68L constructs as indicated. Cell lysates were incubated with immobilized RBP1 RBD. Pull-downs were subjected to SDS-PAGE and detected by WB with anti-FLAG antibody. Lysates represent 2% of total protein from a 10-cm plate of COS-1 cells.

RalGEF protein rather than the RalGAP .

The RalGDS family members are RalGEF proteins that contain a Ras binding domain (RBD), which interacts with active Ras GTPases [27]. The interaction between RalGDS and active Ras is believed to bring the RalGDS in proximity to RalA, causing activation of the small GTPase [28, 29]. It is therefore possible that Rab10 may regulate RalA by alternating the intracellular localization of a RalGEF. To test this hypothesis, we took advantage of a RalA ΔCAAX mutant, in which the C-terminal targeting sequence has been deleted, thus disrupting its intracellular localization [7]. As indicated in **Figure 3.4**, while Rab10 expression significantly activates wild type RalA in COS-1 cells, it was without effect on the activity of the RalA ΔCAAX mutant, demonstrating that the activation of RalA by Rab10 is localization dependent.

AS160 regulates RalA activity through Rab10

As discussed in Chapter 2, our data suggest that Rab10 is a functional target of the GAP protein AS160. Thus, placing RalA downstream of Rab10 prompted us to examine whether AS160 might also regulate RalA activity through Rab10. To test this posibility, we expressed the AS160 GAP domain in COS-1 cells, and measured RalA activity with the RalBP1 pull-down assay. As shown in **Figure 3.5**, overexpressed AS160 GAP domain caused a mild but significant decrease in RalA activity. However, overexpression of the AS160GAP-Rab10tail chimera produced stronger inhibition, presumably due to more specifically targeting the intracellular Rab10, as the chimeric protein contains the Rab10 C-terminal sequence that targets specifically to intracellular compartments where

Figure 3.4: Rab10 activates RalA in a localization dependent manner. COS-1 cells were transiently transfected with FLAG-RalA WT, RalA ΔCAAX and HA-Rab10 Q68L constructs as indicated. Cell lysates were incubated with immobilized RBP1 RBD. Associated proteins were separated by SDS-PAGE and detected by WB with anti-FLAG antibody. Lysates represent 2% of total protein from a 10-cm plate of COS-1 cells.
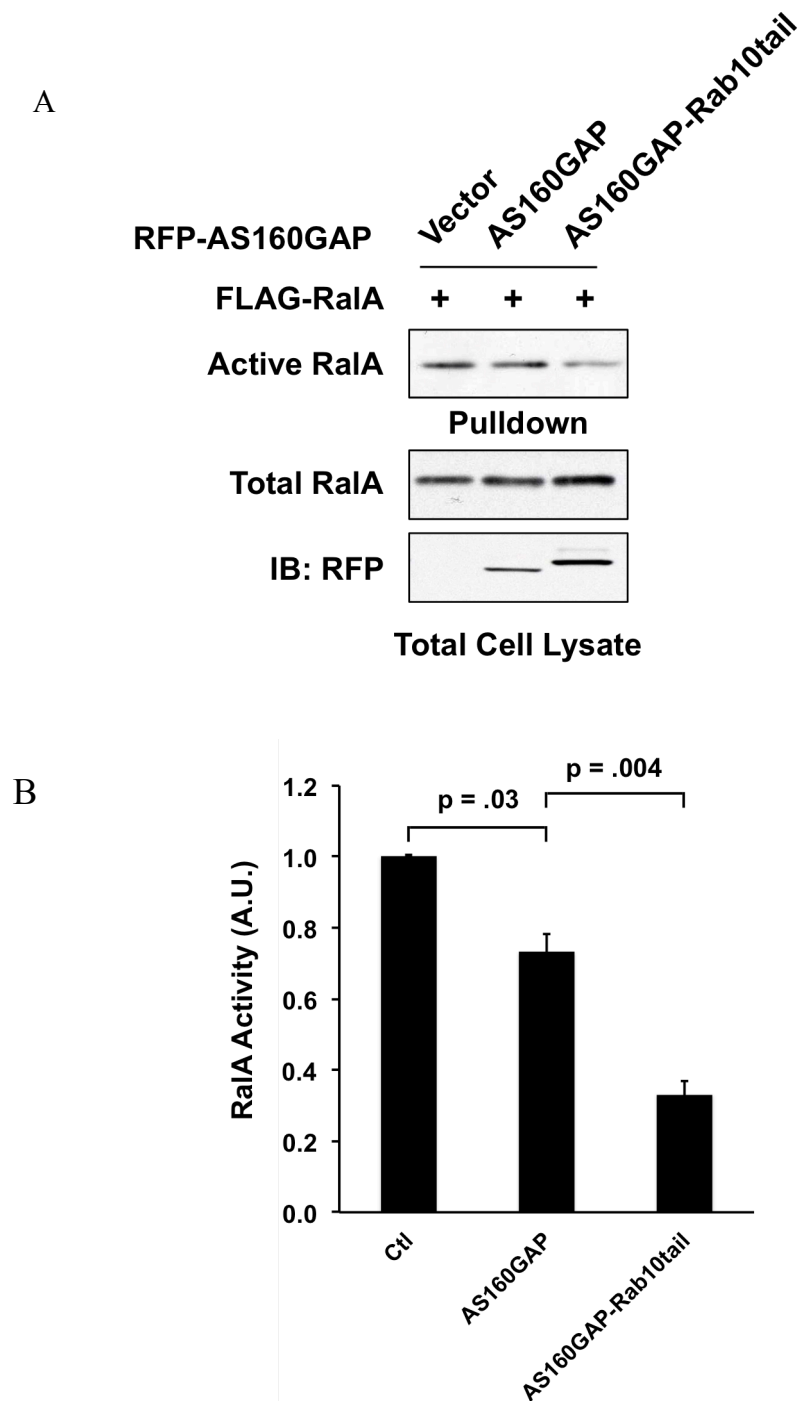
Figure 3.5: AS160 negatively regulates RalA activity through Rab10. A) COS-1 cells were transiently transfected with FLAG-RalA RFP vector, RFP-AS160GAP or RFP-AS160GAP-Rab10tail constructs as indicated. Cell lysates were incubated with immobilized RBP1 RBD. Associated proteins were subjected to SDS-PAGE and determined by WB with anti-FLAG antibody. Lysates represent 2% of total protein from a 10-cm plate of COS-1 cells. B) Quantification of RalA activity in response to AS160GAP or AS160GAP-Rab10tail from three independent experiments as described before. Significance was determined by Student's *t* test.

Rab10 resides. These data suggest that the AS160-Rab10 axis acts as an upstream regulator of RalA activity.

Discussion

Data described here suggest an interesting connection between the two small GTPases Rab10 and RalA, in which Rab10 plays an important role upstream of RalA activation, potentially through recruiting a RalGEF. There are two major possible mechanisms by which this may occur: 1) Rab10 regulates the catalytic activity of RalGEF, or 2) Rab10 regulates the accessibility of RalGEF to RalA. Our data indicate that active Rab10 may recruit a RalGEF to RalA-residing compartments to activate RalA. This recruitment could be achieved through direct interaction, in which the RalGEF might contain a Rab-binding domain and act as a direct effector of Rab10. It is also possible that Rab10 may translocate RalGEF through a bridging adaptor protein, modify the lipid-binding capacity, or even modulate the phosphorylation of the RalGEF.

Numerous cellular processes such as vesicle endocytosis and exocytosis require multiple small GTPases to work cooperatively, which thus must be coordinately regulated to fulfill their functions in a sequential manner [40]. A classic way to achieve this coordination is through a small GTPase cascade, in which a GEF activating a downstream small GTPase interacts with an upstream small GTPase, leading to a sequential activation of small GTPases. This principle is evolutionarily conserved. For instance, the first reported Rab GTPase cascade in yeast was discovered through analysis

of a yeast secretory pathway, in which active Ypt32p (a Rab11 homolog) recruits a RabGEF Sec2p to activate Sec4p (a Rab8/10/13 homolog) to regulate secretion of Golgi-derived vesicles [41, 42]. The most notable small GTPase cascade in human is the Ras-Ral cascade as discussed previously, in which the RalGDS family members are recruited as Ras effectors to activate Ral on the plasma membrane [27]. Our data present a novel small GTPase cascade involving the Rab GTPase Rab10 and the Ras GTPase RalA, indicating Rab10 and RalA may work in concert to regulate insulin-stimulated Glut4 vesicle exocytosis.

Materials and Methods

Antibodies

The anti-HA and anti-myc monoclonal antibodies were purchased from Santa Cruz Biotechnology. The anti-FLAG M2 monoclonal antibody was purchased from Sigma-Aldrich. The anti-RalA monoclonal antibody was purchased from BD Transduction Laboratories. The anti-RFP polyclonal antibody was purchased from Abcam. The anti-phospo-Akt (Thr308) and anti-Rab10 polyclonal antibody was purchased from Cell Signaling Technology. The anti-phospho-AS160 (Thr642) was from Millipore.

Expression Constructs

Rab10 cDNA were from lab stock. AS160 cDNA were from Dr. Gustave Lienhard. Point mutants of Rab10 were created using the QuikChange method (Stratagene). RalA cDNA and mutants are obtained as described in previous studies [12, 43]. As described in chapter 3, to make the AS160GAP-Rab10tail chimera, the GAP domain of AS160 was amplified by PCR with a 3' primer containing the last 132 bp of Rab10 cDNA, and cloned into the RFP construct described before [12]. Automatic DNA sequencing was performed at the University of Michigan DNA Sequencing Core (Ann Arbor, MI). All constructs were sequenced to confirm reading frame.

Cell culture, transfections and siRNA-mediated knockdown

COS-1 cells were maintained in DMEM containing 25mM glucose, 10% fetal bovine serum at 37°C with 5% CO_2. COS-1 cells were transfected with DNA using FuGENE6 transfection reagent (Roche, Indianapolis, IN). 3T3-L1 preadipocytes were cultured in DMEM containing 25 mM glucose, 10% neonatal calf serum at 37°C with 5% CO_2. Confluent cultures were induced to differentiate 3T3-L1 preadipocytes into adipocytes as previously described [44]. Differentiated 3T3-L1 adipocytes were transfected with Stealth siRNA (Invitrogen) by electroporation as previously described [45]. Briefly, adipocytes were put into suspension by mild trypsinization and electroporated with various amounts of oligos under low-voltage conditions (160V, 950 μF).

Immunoprecipitation and immunoblotting

Cell were washed three times with ice-cold PBS (phosphate-buffered saline) and then lysed on ice for 10 min with 1 ml lysis buffer (100 mM Tris, pH 7.5, 1% NP-40, 130 mM NaCl, 1 mM Na_3VO_4, 5 mM $MgCl_2$, 1 mM EDTA, and 10 mM NaF) with Complete, EDTA-free protease inhibitor tablets (Roche). Whole cell lysates were centrifuged for 10 min at $13,000 \times g$. Lysates were incubated with 4 μg of the indicated antibody for 2 h at 4°C, and then protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added for 2 h at 4°C to precipitate the antibody. Beads were washed three times in lysis buffer and then resuspended in 2X SDS sample buffer. The immunoprecipitates were subjected to SDS-PAGE and western blotting was performed using the indicated antibodies.

RalA pull-down activity assay

COS-1 cells or 3T3L-1 adipocytes treated with vehicle or insulin were lysed with lysis buffer (25 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol, 1% NP-40, 5 mM $MgCl_2$, and 1 mM DTT) supplemented with Complete, EDTA-free protease inhibitor tablets and with 10 μM GDP; or 10 μM GTPγS if indicated. Cell lysates were incubated with either GST alone or Agarose-conjugated RalBP1 (Millipore) for 35 min at 4°C. The beads were washed four times with lysis buffer, resuspended in 2X sample buffer, subjected to SDS-PAGE, and analyzed by immunoblotting.

2-Deoxyglucose uptake assay

2-deoxyglucose uptake assay was performed as previously described [46]. Briefly, 3T3-L1 adipocytes were serum starved for 3 hours followed by 30-minute insulin incubation with indicated doses. Glucose uptake was monitored by adding 2-[^{14}C] deoxyglucose for 5 min. The reaction was then quenched with an excess of cold 2-deoxyglucose. Cells were washed with PBS three times and lysed in PBS. Lysate radioactivity was counted using a Beckman scintillation counter.

References:

1.   Bryant, N.J., R. Govers, and D.E. James, *Regulated transport of the glucose transporter GLUT4.* Nat Rev Mol Cell Biol, 2002. **3**(4): p. 267-77.

2.   Watson, R.T., M. Kanzaki, and J.E. Pessin, *Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes.* Endocr Rev, 2004. **25**(2): p. 177-204.

3.   Hou, J.C. and J.E. Pessin, *Ins (endocytosis) and outs (exocytosis) of GLUT4 trafficking.* Curr Opin Cell Biol, 2007. **19**(4): p. 466-73.

4.   Kanzaki, M. and J.E. Pessin, *Insulin signaling: GLUT4 vesicles exit via the exocyst.* Curr Biol, 2003. **13**(14): p. R574-6.

5.   Antonescu, C.N., et al., *Ready, set, internalize: mechanisms and regulation of GLUT4 endocytosis.* Biosci Rep, 2009. **29**(1): p. 1-11.

6.   Grosshans, B.L., D. Ortiz, and P. Novick, *Rabs and their effectors: achieving specificity in membrane traffic.* Proc Natl Acad Sci U S A, 2006. **103**(32): p. 11821-7.

7.   Takai, Y., T. Sasaki, and T. Matozaki, *Small GTP-binding proteins.* Physiol Rev, 2001. **81**(1): p. 153-208.

8.   Cai, H., K. Reinisch, and S. Ferro-Novick, *Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle.* Dev Cell, 2007. **12**(5): p. 671-82.

9.   Novick, P., et al., *Interactions between Rabs, tethers, SNAREs and their regulators in exocytosis.* Biochem Soc Trans, 2006. **34**(Pt 5): p. 683-6.

10.   Saltiel, A.R. and J.E. Pessin, *Insulin signaling in microdomains of the plasma membrane.* Traffic, 2003. **4**(11): p. 711-6.

11.   Welsh, G.I., et al., *Regulation of small GTP-binding proteins by insulin.* Biochem Soc Trans, 2006. **34**(Pt 2): p. 209-12.

12.   Chen, X.W., et al., *Activation of RalA is required for insulin-stimulated Glut4 trafficking to the plasma membrane via the exocyst and the motor protein Myo1c.* Dev Cell, 2007. **13**(3): p. 391-404.

13.   Ishikura, S., A. Koshkina, and A. Klip, *Small G proteins in insulin action: Rab and Rho families at the crossroads of signal transduction and GLUT4 vesicle traffic.* Acta Physiol (Oxf), 2008. **192**(1): p. 61-74.

14.   Sano, H., et al., *Rab10, a target of the AS160 Rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane.* Cell Metab, 2007. **5**(4): p. 293-303.

15.   Sano, H., et al., *Rab10 in insulin-stimulated GLUT4 translocation.* Biochem J, 2008. **411**(1): p. 89-95.

16.   Bos, J.L., H. Rehmann, and A. Wittinghofer, *GEFs and GAPs: critical elements in the control of small G proteins.* Cell, 2007. **129**(5): p. 865-77.

17.   Watson, R.T. and J.E. Pessin, *Bridging the GAP between insulin signaling and GLUT4 translocation.* Trends Biochem Sci, 2006. **31**(4): p. 215-22.

18.   Chen, X.W., et al., *A Ral GAP complex links PI 3-kinase/Akt signaling to RalA activation in insulin action.* Mol Biol Cell, 2011. **22**(1): p. 141-52.

19.   Shirakawa, R., et al., *Tuberous sclerosis tumor suppressor complex-like complexes act as GTPase-activating proteins for Ral GTPases.* J Biol Chem, 2009. **284**(32): p. 21580-8.

20.   de Bruyn, K.M., et al., *RalGEF2, a pleckstrin homology domain containing guanine nucleotide exchange factor for Ral.* J Biol Chem, 2000. **275**(38): p. 29761-6.

21.   Kikuchi, A., et al., *ralGDS family members interact with the effector loop of ras p21*. Mol Cell Biol, 1994. **14**(11): p. 7483-91.

22.   Peterson, S.N., et al., *Identification of a novel RalGDS-related protein as a candidate effector for Ras and Rap1*. J Biol Chem, 1996. **271**(47): p. 29903-8.

23.   Rebhun, J.F., H. Chen, and L.A. Quilliam, *Identification and characterization of a new family of guanine nucleotide exchange factors for the ras-related GTPase Ral*. J Biol Chem, 2000. **275**(18): p. 13406-10.

24.   Shao, H. and D.A. Andres, *A novel RalGEF-like protein, RGL3, as a candidate effector for rit and Ras*. J Biol Chem, 2000. **275**(35): p. 26914-24.

25.   Wolthuis, R.M., et al., *RalGDS-like factor (Rlf) is a novel Ras and Rap 1A-associating protein*. Oncogene, 1996. **13**(2): p. 353-62.

26.   Ceriani, M., et al., *Functional analysis of RalGPS2, a murine guanine nucleotide exchange factor for RalA GTPase*. Exp Cell Res, 2007. **313**(11): p. 2293-307.

27.   Ferro, E. and L. Trabalzini, *RalGDS family members couple Ras to Ral signalling and that's not all*. Cell Signal, 2010. **22**(12): p. 1804-10.

28.   Kishida, S., et al., *Colocalization of Ras and Ral on the membrane is required for Ras-dependent Ral activation through Ral GDP dissociation stimulator*. Oncogene, 1997. **15**(24): p. 2899-907.

29.   Matsubara, K., et al., *Plasma membrane recruitment of RalGDS is critical for Ras-dependent Ral activation*. Oncogene, 1999. **18**(6): p. 1303-12.

30.   Eguez, L., et al., *Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein*. Cell Metab, 2005. **2**(4): p. 263-72.

31.   Bodemann, B.O. and M.A. White, *Ral GTPases and cancer: linchpin support of the tumorigenic platform*. Nat Rev Cancer, 2008. **8**(2): p. 133-40.

32.   Neel, N.F., et al., *The RalGEF-Ral Effector Signaling Network: The Road Less Traveled for Anti-Ras Drug Discovery*. Genes Cancer, 2011. **2**(3): p. 275-87.

33.   Wolthuis, R.M., et al., *Stimulation of gene induction and cell growth by the Ras effector Rlf*. EMBO J, 1997. **16**(22): p. 6748-61.

34.   Camonis, J.H. and M.A. White, *Ral GTPases: corrupting the exocyst in cancer cells*. Trends Cell Biol, 2005. **15**(6): p. 327-32.

35.   Inoue, M., et al., *The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin*. Nature, 2003. **422**(6932): p. 629-33.

36.   Lim, K.H., et al., *Activation of RalA is critical for Ras-induced tumorigenesis of human cells.* Cancer Cell, 2005. **7**(6): p. 533-45.

37.   Moskalenko, S., et al., *The exocyst is a Ral effector complex.* Nat Cell Biol, 2002. **4**(1): p. 66-72.

38.   Sugihara, K., et al., *The exocyst complex binds the small GTPase RalA to mediate filopodia formation.* Nat Cell Biol, 2002. **4**(1): p. 73-8.

39.   Peng, W., et al., *Structural study of the Cdc25 domain from Ral-specific guanine-nucleotide exchange factor RalGPS1a.* Protein Cell, 2011. **2**(4): p. 308-19.

40.   Mizuno-Yamasaki, E., F. Rivera-Molina, and P. Novick, *GTPase Networks in Membrane Traffic.* Annu Rev Biochem, 2012.

41.   Walch-Solimena, C., R.N. Collins, and P.J. Novick, *Sec2p mediates nucleotide exchange on Sec4p and is involved in polarized delivery of post-Golgi vesicles.* J Cell Biol, 1997. **137**(7): p. 1495-509.

42.   Ortiz, D., et al., *Ypt32 recruits the Sec4p guanine nucleotide exchange factor, Sec2p, to secretory vesicles; evidence for a Rab cascade in yeast.* J Cell Biol, 2002. **157**(6): p. 1005-15.

43.   Chen, X.W., et al., *RalA-exocyst-dependent recycling endosome trafficking is required for the completion of cytokinesis.* J Biol Chem, 2006. **281**(50): p. 38609-16.

44.   Elmendorf, J.S., D. Chen, and J.E. Pessin, *Guanosine 5'-O-(3-thiotriphosphate) (GTPgammaS) stimulation of GLUT4 translocation is tyrosine kinase-dependent.* J Biol Chem, 1998. **273**(21): p. 13289-96.

45.   Inoue, M., et al., *Compartmentalization of the exocyst complex in lipid rafts controls Glut4 vesicle tethering.* Mol Biol Cell, 2006. **17**(5): p. 2303-11.

46.   Baumann, C.A., et al., *CAP defines a second signalling pathway required for insulin-stimulated glucose transport.* Nature, 2000. **407**(6801): p. 202-7.

Chapter 4

Perspectives and Future Directions

Impaired insulin-stimulated glucose transport into muscle and fat cells is one of the early defects of type 2 diabetes [1, 2]. Insulin promotes glucose uptake by recruiting the facilitative glucose transporter Glut4 from intracellular vesicles to the plasma membrane, a process of signaling-regulated vesicle exocytosis. While it is clear that trafficking of Glut4 vesicle is orchestrated by insulin signaling cascades in concert with vesicle transport machineries, the precise mechanisms by which these signaling events regulate the transport machineries remain uncertain [3, 4]. Nonetheless, extensive studies of the superfamily of small GTPases, and their upstream regulators GAPs (GTPase-activating proteins) and GEFs (guanine nucleotide exchange factors), suggest that these proteins may play a vital role at the intersection of insulin signaling and vesicle trafficking. Small GTPases function as nucleotide-dependent switches that recruit sets of when active. GAPs and GEFs are mostly multi-domain proteins, which fine-tune the activity status of target small GTPase in response to upstream signaling inputs. The focus of my dissertation work was to characterize the roles and regulatory mechanisms of some of these small GTPases and their regulators in insulin action and Glut4 trafficking.

In chapter 2 of this thesis, we characterized Rab10, a Rab GTPase required for insulin-stimulated Glut4 translocation [5, 6], as a functional target of the Akt substrate and RabGAP protein AS160 [7, 8]. Our work also suggested that the GAP activity of AS160 towards Rab10 is negatively regulated by Akt phosphorylation (**Figure 4.1**). This

resembles the scenarios in which Akt-catalyzed phosphorylation negatively regulates the GAP activity of TSC2 toward the small GTPase Rheb [9, 10], and RGC2 towards RalA [11]. Despite intensive investigation, the exact mechanisms by which Akt regulates TSC2 and RGC2 remain uncertain [11, 12]. One major unaddressed question in our current study concerns how Akt precisely regulates the GAP activity of AS160.

For TSC2 [10], RGC2 [13] and AS160 [7], the identified Akt phosphorylation sites are found outside of the GAP catalytic domain, rendering it less likely that phosphorylation may directly regulate catalytic activity. In addition, although it has not been tested *in vivo*, studies have suggested that the *in vitro* GAP activity of TSC2 towards Rheb [14], or RGC2 towards RalA [11], is not affected by Akt phosphorylation. These findings thus favor another potential regulatory paradigm, in which Akt phosphorylation may lead to translocation of the GAP proteins, partitioning them from their substrate small GTPases. Most small GTPases contain a special targeting sequence at their C-terminal region that undergoes lipid modification to localizes the protein to a distinct membrane compartment [15]. The functionalities of these membrane structures are defined by the resident small GTPases through recruiting and releasing different sets of effectors [16]. It is therefore possible that GAP proteins may also be recruited to or sequestered from these membrane structures to regulate the activity of their cognate small GTPases.

One approach to test this 'translocation model' is to track the subcellular localization of AS160 under different conditions, which will give the most direct evidence of whether Akt phosphorylation would induce translocation of AS160. One previous study has

reported that with immunoprecipitation and immunoblotting, AS160 is found to be associated with Glut4-containing vesicles in 3T3-L1 adipocytes; and while insulin stimulation leads to reduced association, pretreating the cells with PI 3-kinase wortmannin could reverse the effect of insulin [17]. However, it is noteworthy that the exact definition and precise markers of Glut4 vesicles are still under debate, due to the highly dynamic nature of these vesicles. Another caveat of the localization studies would be the sensitivity of current techniques, which may not be able to detect the translocation if it occurred between two highly adjacent and related membrane compartments.

Another approach that may address the above-mentioned issues is to take advantage of the biochemical feature of the small GTPases. As discussed before, different small GTPases contain different targeting sequence at their C-terminal region, which undergoes distinct lipid modification and is able to insert each small GTPase into the bilayer of its designated membrane compartment [15]. Therefore, a chimeric protein with the Rab10 targeting sequence attached to AS160 could not only target to the Rab10-containing membrane compartments, but also escape any potential translocation regulation. This chimeric protein (AS160-Rab10tail) would thus be a useful tool to discriminate whether Akt phosphorylation regulates AS160 through catalytic inhibition or through a translocation-related mechanism. By comparing the GAP activity of AS160 and AS160-Rab10tail in response to Akt phosphorylation, information could be obtained regarding the precise mechanism by which the kinase regulates its substrate GAP proteins.

Another unaddressed question in this study concerns the sensitivity of Rab10 to insulin. Whereas activation of Akt resulted in increased activity of Rab10 (**Figure 2.4C and D**), Rab10 activation by insulin was not detected by the effector pull-down assay, despite multiple attempts (data not shown). Possible explanations for this paradox include that insulin may only activate a fraction of Rab10 or that Rab10 may undergo rapid cycling from activation to inactivation. These scenarios may be evaluated by more sophisticated assays such as a FRET (Fluorescence resonance energy transfer) assay [18], which could be developed by tagging Rab10 with a donor fluorescent probe and Rim1 Rab-binding domain with an acceptor fluorescent probe. This potential assay may be used to observe activation of Rab10 in real-time in the living cell, enabling us to study the temporal and spatial regulation of Rab10 in response to insulin.

In chapter 3 of this thesis, our results present an interesting crosstalk between Rab10 GTPase and RalA GTPase, showing that Rab10 is potentially involved in insulin-stimulated RalA activation, likely through a yet-to-be-identified RalGEF (**Figure 4.1**). Previous work has suggested that insulin-stimulated RalA activation involves the inhibition of the RalGAP RGC2 by Akt phosphorylation [11]. Our current data suggest that in addition to RGC2, a RalGEF may also be implicated in insulin-stimulated RalA activation. Although it is still not understood why two positive signals would be required for RalA activation, one reasonable explanation is that activation of the RalGEF may be necessary to initiate RalA function, while inactivation of RGC2 may be essential for sustained activation of RalA and the continuity of downstream events. Therefore, the GEF and GAP cascades may work in a coordinated fashion, to promote spatially and

temporally regulated RalA activity and achieve the fine-tuned control of Glut4 vesicle translocation.

Two immediate follow up questions to this study will be: 1) What is the identity of this Rab10-sensitive RalGEF? 2) How does Rab10 regulate the activity of this RalGEF? To date, at least six different members have been identified in the RalGEF family, implicated in different cellular processes in various cell types [19-25]. Considering the possibility of other unidentified RalGEFs, one feasible approach to determine the identity of the RalGEF potentially involved in the Rab10 and RalA cascade is to combine a proteomics analysis with a specific RalA mutant that preferentially interacts with GEF proteins. Alternatively, a proteomics analysis of the potential Rab10 effectors isolated with the constitutively active Rab10 mutant may also be helpful in pinpointing this RalGEF.

Similar to the regulation of a GAP protein, there are two major possible mechanisms by which upstream signaling could regulate a GEF protein: direct regulation on its catalytic activity, or control of its accessibility to its small GTPase substrate. The translocation paradigm is commonly seen in the regulation of GEF proteins, with the Ras GEF SOS as the best characterized example. In response to hormonal signaling, SOS is recruited to the plasma membrane where Ras is located, through the adaptor protein Grb2, which interacts with both SOS and the tyrosine-phosphorylated-receptors [26]. This translocation is terminated by ERK-mediated phosphorylation on SOS, which results in dissociation of SOS from Grb2, serving as a negative feedback control of the SOS-Ras-ERK pathway [27]. Another classic way of regulating GEF translocation is through direct

interaction with an upstream GTP-bound small GTPase, leading to a cascade activation of the downstream small GTPase [28]. Therefore, there are several possibilities of how Rab10 could regulate the potential RalGEF activity towards RalA, such as direct interaction, recruitment of adaptor proteins, or kinase-mediated phosphorylation. Understanding the exact mechanism would rely on characterizing the biochemical feature of the RalGEF protein.
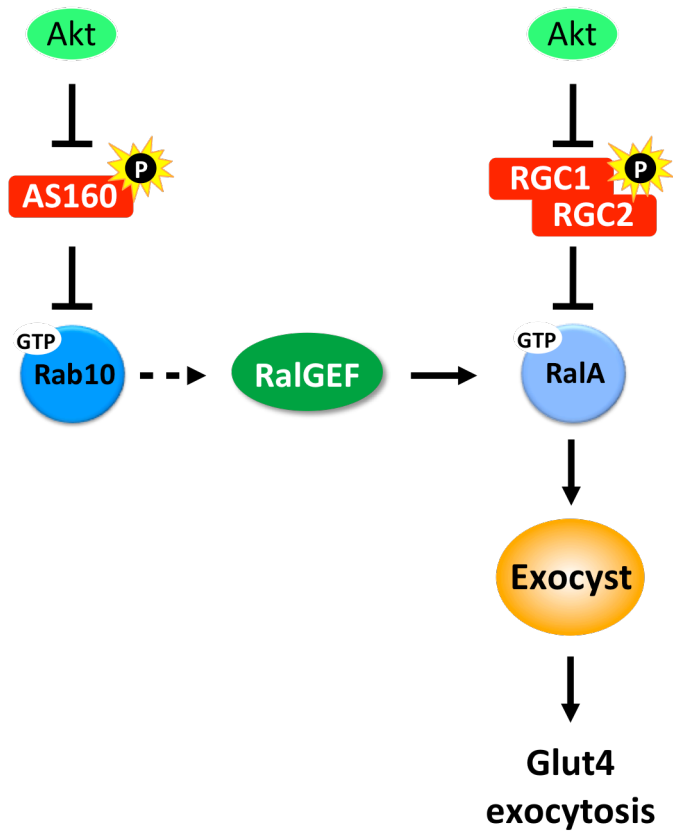
Figure 4.1: Schematic of cascade activation of small GTPases in insulin-stimulated Glut4 exocytosis. Activation of the RalA GTPase requires phosphorylation and inhibition of the GAP complex RGC1/2 by Akt signaling [11]. In addition, Akt signaling activates the Rab10 GTPase by directly phosphorylating and inhibiting the Rab10 GAP AS160. Active Rab10 can activate RalA via recruitment of an as-yet-unidentified GEF of RalA. This cascading activation of small GTPases eventually promotes Glut4 exocytosis through engagement of the RalA effector exocyst complex [29].

References:

1. Saltiel, A.R. and C.R. Kahn, *Insulin signalling and the regulation of glucose and lipid metabolism.* Nature, 2001. **414**(6865): p. 799-806.
2. Watson, R.T., M. Kanzaki, and J.E. Pessin, *Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes.* Endocr Rev, 2004. **25**(2): p. 177-204.
3. Huang, S. and M.P. Czech, *The GLUT4 glucose transporter.* Cell Metab, 2007. **5**(4): p. 237-52.
4. Hou, J.C. and J.E. Pessin, *Ins (endocytosis) and outs (exocytosis) of GLUT4 trafficking.* Curr Opin Cell Biol, 2007. **19**(4): p. 466-73.
5. Sano, H., et al., *Rab10, a target of the AS160 Rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane.* Cell Metab, 2007. **5**(4): p. 293-303.
6. Sano, H., et al., *Rab10 in insulin-stimulated GLUT4 translocation.* Biochem J, 2008. **411**(1): p. 89-95.
7. Sano, H., et al., *Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation.* J Biol Chem, 2003. **278**(17): p. 14599-602.
8. Miinea, C.P., et al., *AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain.* Biochem J, 2005. **391**(Pt 1): p. 87-93.
9. Li, Y., K. Inoki, and K.L. Guan, *Biochemical and functional characterizations of small GTPase Rheb and TSC2 GAP activity.* Mol Cell Biol, 2004. **24**(18): p. 7965-75.
10. Manning, B.D. and L.C. Cantley, *Rheb fills a GAP between TSC and TOR.* Trends Biochem Sci, 2003. **28**(11): p. 573-6.
11. Chen, X.W., et al., *A Ral GAP complex links PI 3-kinase/Akt signaling to RalA activation in insulin action.* Mol Biol Cell, 2011. **22**(1): p. 141-52.
12. Huang, J. and B.D. Manning, *A complex interplay between Akt, TSC2 and the two mTOR complexes.* Biochem Soc Trans, 2009. **37**(Pt 1): p. 217-22.
13. Gridley, S., et al., *Adipocytes contain a novel complex similar to the tuberous sclerosis complex.* Cell Signal, 2006. **18**(10): p. 1626-32.
14. Cai, S.L., et al., *Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning.* J Cell Biol, 2006. **173**(2): p. 279-89.
15. Takai, Y., T. Sasaki, and T. Matozaki, *Small GTP-binding proteins.* Physiol Rev, 2001. **81**(1): p. 153-208.
16. Mizuno-Yamasaki, E., F. Rivera-Molina, and P. Novick, *GTPase Networks in Membrane Traffic.* Annu Rev Biochem, 2012.
17. Larance, M., et al., *Characterization of the role of the Rab GTPase-activating protein AS160 in insulin-regulated GLUT4 trafficking.* J Biol Chem, 2005. **280**(45): p. 37803-13.

18. Padilla-Parra, S. and M. Tramier, *FRET microscopy in the living cell: Different approaches, strengths and weaknesses.* Bioessays, 2012. **34**(5): p. 369-76.
19. Kikuchi, A., et al., *ralGDS family members interact with the effector loop of ras p21.* Mol Cell Biol, 1994. **14**(11): p. 7483-91.
20. Peterson, S.N., et al., *Identification of a novel RalGDS-related protein as a candidate effector for Ras and Rap1.* J Biol Chem, 1996. **271**(47): p. 29903-8.
21. Wolthuis, R.M., et al., *RalGDS-like factor (Rlf) is a novel Ras and Rap 1A-associating protein.* Oncogene, 1996. **13**(2): p. 353-62.
22. de Bruyn, K.M., et al., *RalGEF2, a pleckstrin homology domain containing guanine nucleotide exchange factor for Ral.* J Biol Chem, 2000. **275**(38): p. 29761-6.
23. Rebhun, J.F., H. Chen, and L.A. Quilliam, *Identification and characterization of a new family of guanine nucleotide exchange factors for the ras-related GTPase Ral.* J Biol Chem, 2000. **275**(18): p. 13406-10.
24. Shao, H. and D.A. Andres, *A novel RalGEF-like protein, RGL3, as a candidate effector for rit and Ras.* J Biol Chem, 2000. **275**(35): p. 26914-24.
25. Ceriani, M., et al., *Functional analysis of RalGPS2, a murine guanine nucleotide exchange factor for RalA GTPase.* Exp Cell Res, 2007. **313**(11): p. 2293-307.
26. Aronheim, A., et al., *Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway.* Cell, 1994. **78**(6): p. 949-61.
27. Waters, S.B., K. Yamauchi, and J.E. Pessin, *Insulin-stimulated disassociation of the SOS-Grb2 complex.* Mol Cell Biol, 1995. **15**(5): p. 2791-9.
28. Bos, J.L., H. Rehmann, and A. Wittinghofer, *GEFs and GAPs: critical elements in the control of small G proteins.* Cell, 2007. **129**(5): p. 865-77.
29. Chen, X.W., et al., *Activation of RalA is required for insulin-stimulated Glut4 trafficking to the plasma membrane via the exocyst and the motor protein Myo1c.* Dev Cell, 2007. **13**(3): p. 391-404.