Supplementary Information

Supplementary Experimental Procedures

Antibodies The anti-Actin monoclonal antibody, anti-FLAG M2 monoclonal antibody, anti-GARNL1 polyclonal antibody, and rabbit IgG polyclonal antibody were purchased from Sigma-Aldrich. The anti-pTyr (4G10) polyclonal antibody was purchased from Upstate Cell Signaling Solutions. The anti-pAkt (S473) polyclonal antibody, anti-PPARγ polyclonal antibody, anti-p-Akt Substrate polyclonal antibody, anti-pan Akt polyclonal antibody, anti-Akt1 monoclonal antibody, and anti-Akt2 polyclonal antibody were purchased from Cell Signaling Technology. The anti-HA monoclonal antibody, anti-pErk polyclonal antibody, and anti-GST monoclonal antibody were purchased from Santa Cruz Biotechnology. The anti-Sec8 monoclonal antibody was purchased from Stressgen. The anti-RalA monoclonal antibody and the anti-Caveolin polyclonal antibody were purchased from BD Biosciences. The anti-Glut4 polyclonal antibody was purchased from Alpha Diagnostics. The anti-Exo70 polyclonal antibody was previously described (Chen *et al.*, 2006).

RNAi Stealth siRNA were ordered from Invitrogen. SiRNA for Akt1 and Akt2 has been described previously (Chang *et al.*, 2007). The following sequences (sense) were used to deplete specific proteins.

Control-1: 5'-CCA CCG AAC CAC UUC UAU ACA GUC C-3'
Control-2: 5'-CCG UAU UCG GUU AGU GGU GAC CUA U-3'

RGC1-1: 5'-UUU AAA UGG AGG AAA UGA AGG ACC G-3'
RGC1-2: 5'-AAG AGU UGU AGA UCU CAG AGA AGU G-3'
RGC1-3: 5'-GAG GUG GUC CUA GAA GGA AAG UUU A-3'

RGC2-1: 5'-GAG AGU CUA UGU GAG GGA UAU CUC A-3'
RGC2-2: 5'-GCA AGA AGA CAA GUG UUC CAU UCU A-3'
RGC2-3: 5'-UUC ACA UUU GUG UUG UCC UCC UCU G-3'

GARNL1-1: 5'-CAG AAA CAA GCA GCC UUA AUC AGC A-3'

TSC2: 5'-CCU GAA UCC UCU CUC UUA AAC UUG A-3'

p120GAP: 5'-UAA ACU GGU UGU UAG GUG UUG GAC A-3'

Rap1GAP: 5'-AAA UCC UGC AAU UUC ACU UUC UGG C-3'

AS160: 5'-GAU AAC GAG GAU GCC UUC UAC AAC U-3'

Supplementary Materials and Methods

Immunoprecipitations, Pulldowns, and Western Blotting were performed as described in Materials and Methods.

In vitro *GAP assays and Kinase assays* were performed as described in Materials and Methods.

***Primary Adipocyte Isolation*.** Primary adipocytes and SVF were isolated as previously described (Liu *et al.*, 2005).

Supplementary References

Chang, L., Chiang, S. H., and Saltiel, A. R. (2007). TC10alpha is required for insulin-stimulated glucose uptake in adipocytes. Endocrinology *148,* 27-33.
Chen, X. W., Leto, D., Chiang, S. H., Wang, Q., and Saltiel, A. R. (2007). Activation of RalA is required for insulin-stimulated Glut4 trafficking to the plasma membrane via the exocyst and the motor protein Myo1c. Dev. Cell *13,* 391-404.
Liu, J., DeYoung, S. M., Zhang, M., Cheng, A., and Saltiel, A. R. (2005). Changes in integrin expression during adipocyte differentiation. Cell Metab. *2,* 165-177.

Supplementary Figure Legends

Figure S1. GTP loading status of wild type and GTPase-dead RalA in 3T3-L1 adipocytes. 3T3-L1 adipocytes were infected with lentivirus expressing either FLAG-tagged RalA wild type or RalA(G23V) (GTPase-dead) as previously described (Chen, 2007). Cell lysates were subjected to a pulldown assay to detect RalA activation. Pulldowns represent the amount of active RalA from half of a 10-cm plate of adipocytes. Lysates represent 2% of total protein from a 10-cm plate of adipocytes. Active RalA was determined by WB. When indicated, 1/4 of the pulldown was loaded for SDS-PAGE for better visualization of RalA by WB.

Figure S2. 3T3-L1 adipocytes were transfected with control siRNA or siRNA to specifically deplete a GAP from 3T3-L1 adipocytes. Four days after transfection, cell lysates were subjected to a pulldown assay to detect RalA activation. Pulldowns represent the amount of active RalA from half of a 10-cm plate of adipocytes. Lysates represent 2% of total protein from a 10-cm plate of adipocytes (A) RalA activity in TSC2-depleted 3T3-L1 adipocytes. (B) RalA activity in p120GAP-depleted 3T3-L1 adipocytes. (C) RalA activity in Rap1GAP-depleted 3T3-L1 adipocytes. (D) RalA activity in AS160-depleted 3T3-L1 adipocytes.

Figure S3. RGC GAP activity is specific for Ral GTPases. (A) Recombinant GST-RalB loaded with γ^{32}-GTP was incubated with immune-complex from a control IgG IP or an anti-RGC2 IP from a 150-mm plate of adipocytes. Hydrolysis of GTP was measured as a reflection of GTPase activity. (B) Recombinant GST-Rheb loaded with γ^{32}-GTP was incubated with immune-complex from a control IgG IP or an anti-RGC2 IP from a 150-mm plate of adipocytes. Hydrolysis of GTP was measured as a reflection of GTPase activity. (C) Recombinant GST-H-Ras loaded with γ^{32}-GTP was incubated with immune-complex from a control IgG IP or an anti-RGC2 IP from a 150-mm plate of adipocytes. Hydrolysis of GTP was measured as a reflection of GTPase activity.

Figure S4. Expression of RGC2 and GARNL1 in adipocytes. (A) ClustalW alignment of the GAP domains of RGC2 and GARNL1. Identical residues are highlighted in red. (B) Total cell lysates were collected at the indicated stages of differentiation. Lysates were resolved by SDS-PAGE and subjected to WB analysis with the indicated antibodies. (C) Adipocytes and the stromal vascular fraction were isolated from mouse epididymal fat pads. Lysates were resolved by SDS-PAGE and subjected to WB analysis with the indicated antibodies.

Figure S5. In vitro phosphorylation of the RGC does not inhibit catalytic activity. Overexpressed RGC proteins were immunoprecipitated from Cos-1 cells with an anti-FLAG antibody and then subjected to an in vitro kinase assay with 100ng of recombinant Akt2 kinase and 100μM ATP. After incubation at 32^{o}C for 30 minutes, the RGC2 immune-complexes were incubated with γ32-GTP-loaded recombinant RalA for 30 minutes. Hydrolysis of GTP was measured as a reflection of GTPase activity.

Figure S1

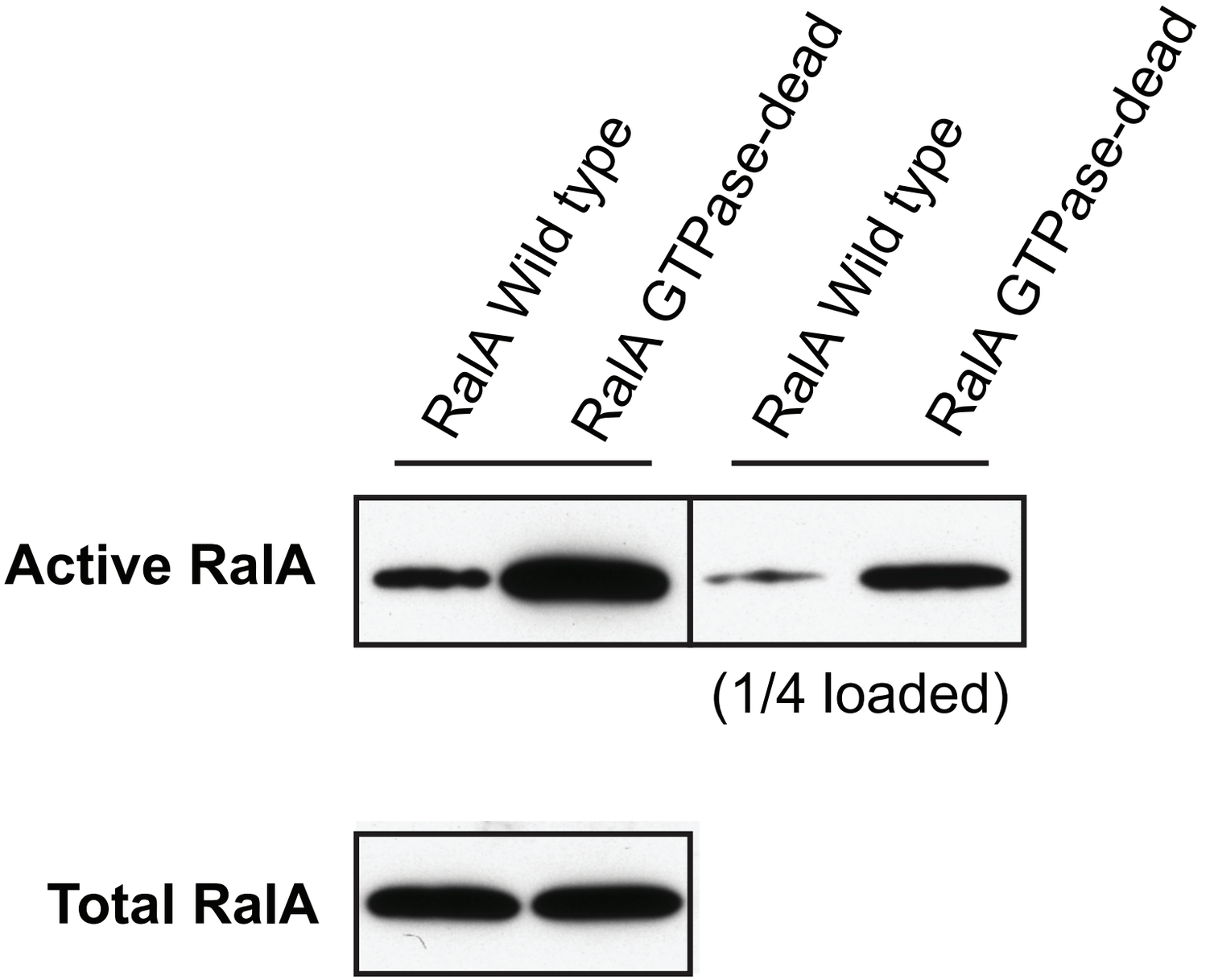

RalA Wild type
RalA GTPase-dead
RalA Wild type
RalA GTPase-dead
Active RalA
(1/4 loaded)
Total RalA

Figure S2

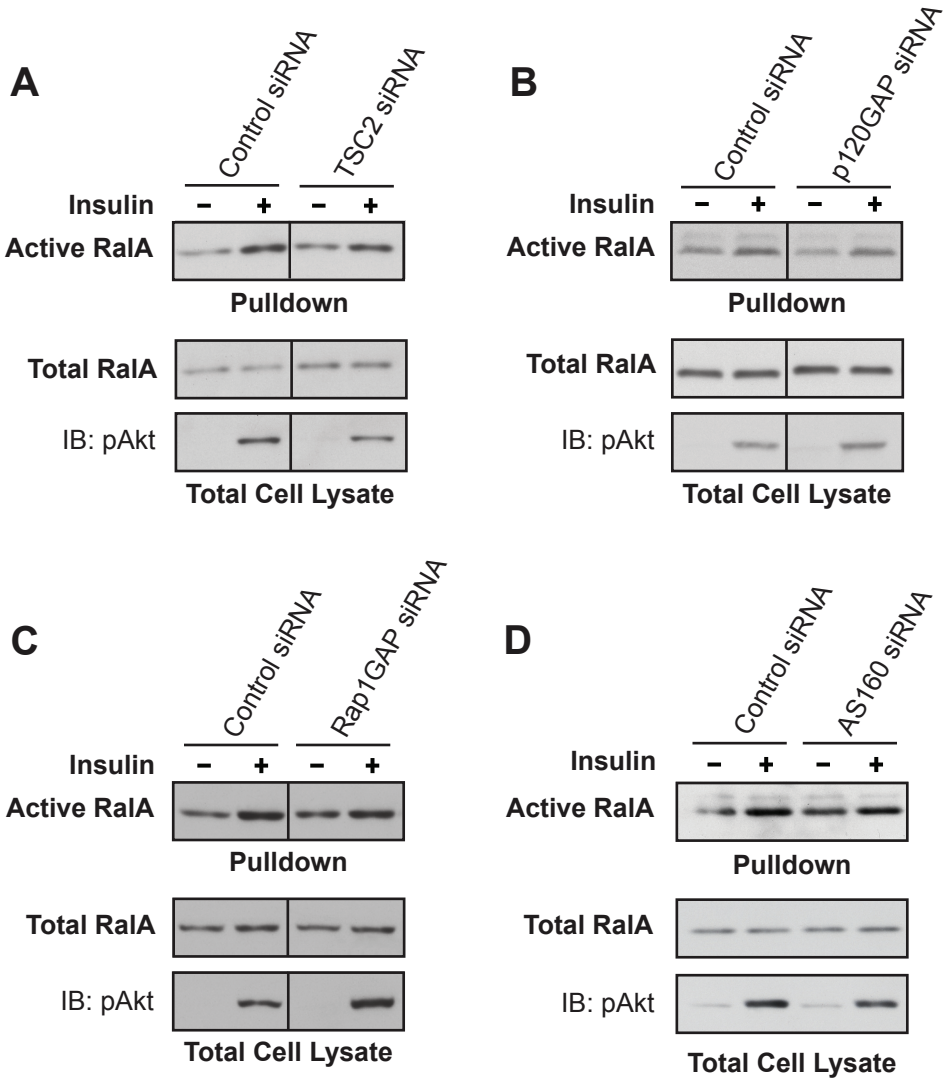

A
Control siRNA
TSC2 siRNA
Insulin
– + – +
Active RalA
Pulldown
Total RalA
IB: pAkt
Total Cell Lysate
B
Control siRNA
p120GAP siRNA
Insulin
– + – +
Active RalA
Pulldown
Total RalA
IB: pAkt
Total Cell Lysate
C
Control siRNA
Rap1GAP siRNA
Insulin
– + – +
Active RalA
Pulldown
Total RalA
IB: pAkt
Total Cell Lysate
D
Control siRNA
AS160 siRNA
Insulin
– + – +
Active RalA
Pulldown
Total RalA
IB: pAkt
Total Cell Lysate

Figure S3

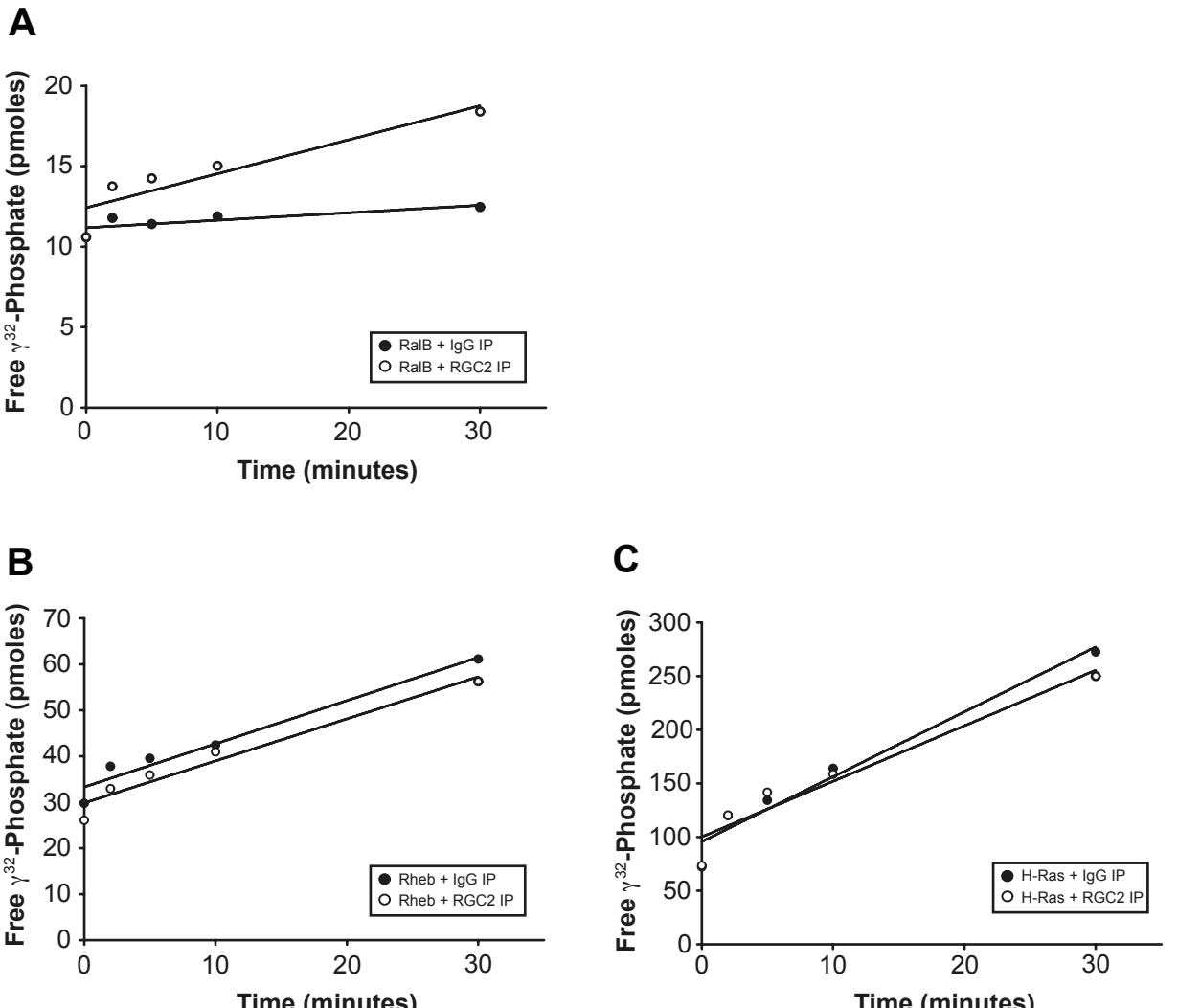
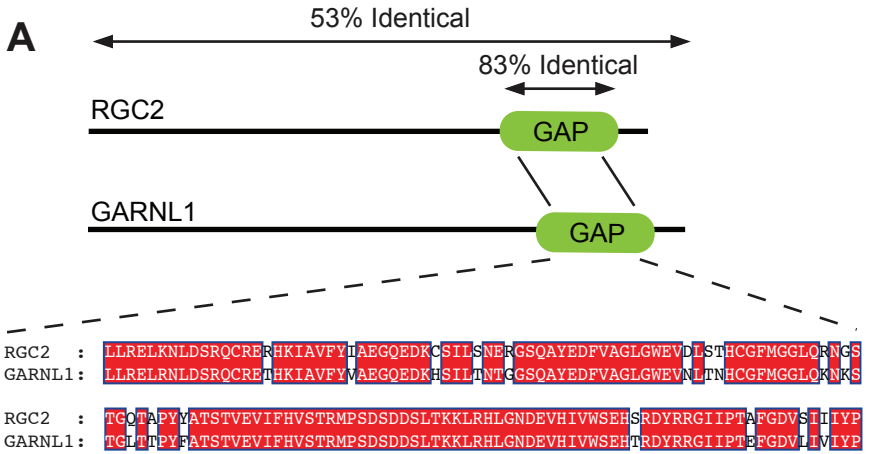
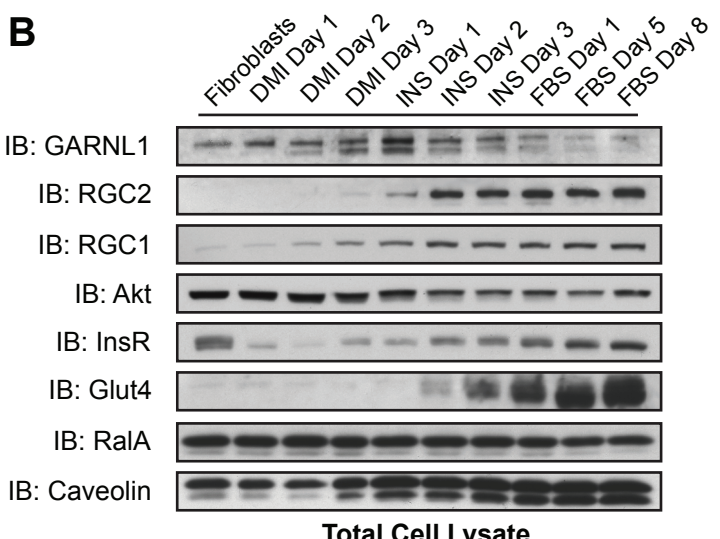

A
Free γ32-Phosphate (pmoles)
20
15
10
5
0
0
10
20
30
Time (minutes)
RalB + IgG IP
RalB + RGC2 IP
B
Free γ32-Phosphate (pmoles)
70
60
50
40
30
20
10
0
0
10
20
30
Time (minutes)
Rheb + IgG IP
Rheb + RGC2 IP
C
Free γ32-Phosphate (pmoles)
300
250
200
150
100
50
0
0
10
20
30
Time (minutes)
H-Ras + IgG IP
H-Ras + RGC2 IP

Figure S4


A
53% Identical
83% Identical
RGC2
GAP
GARNL1
GAP
RGC2 : LLRELKNLDSRQCRERHKIAVFYIAEGQEDKCSILSNERGSQAYEDFVAGLGWEVDLSTHCGFMGGLQRNGS
GARNL1: LLRELRNLDSRQCRETHKIAVFYVAEGQEDKHSILTNTGGSQAYEDFVAGLGWEVNLTNHCGFMGGLQKNKS
RGC2 : TGQTAPYYATSTVEVIFHVSTRMPSDSDDSLTKKLRHLGNDEVHIVWSEHSRDYRRGIIPTAFGDVSITIYP
GARNL1: TGLTTPYFATSTVEVIFHVSTRMPSDSDDSLTKKLRHLGNDEVHIVWSEHTRDYRRGIIPTEFGDVLIVIYP


B
Fibroblasts
DMI Day 1
DMI Day 2
DMI Day 3
INS Day 1
INS Day 2
INS Day 3
FBS Day 1
FBS Day 5
FBS Day 8
IB: GARNL1
IB: RGC2
IB: RGC1
IB: Akt
IB: InsR
IB: Glut4
IB: RalA
IB: Caveolin
Total Cell Lysate

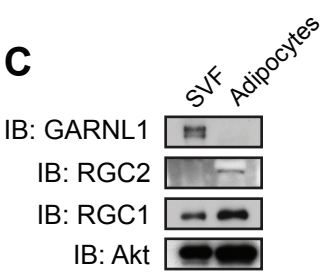
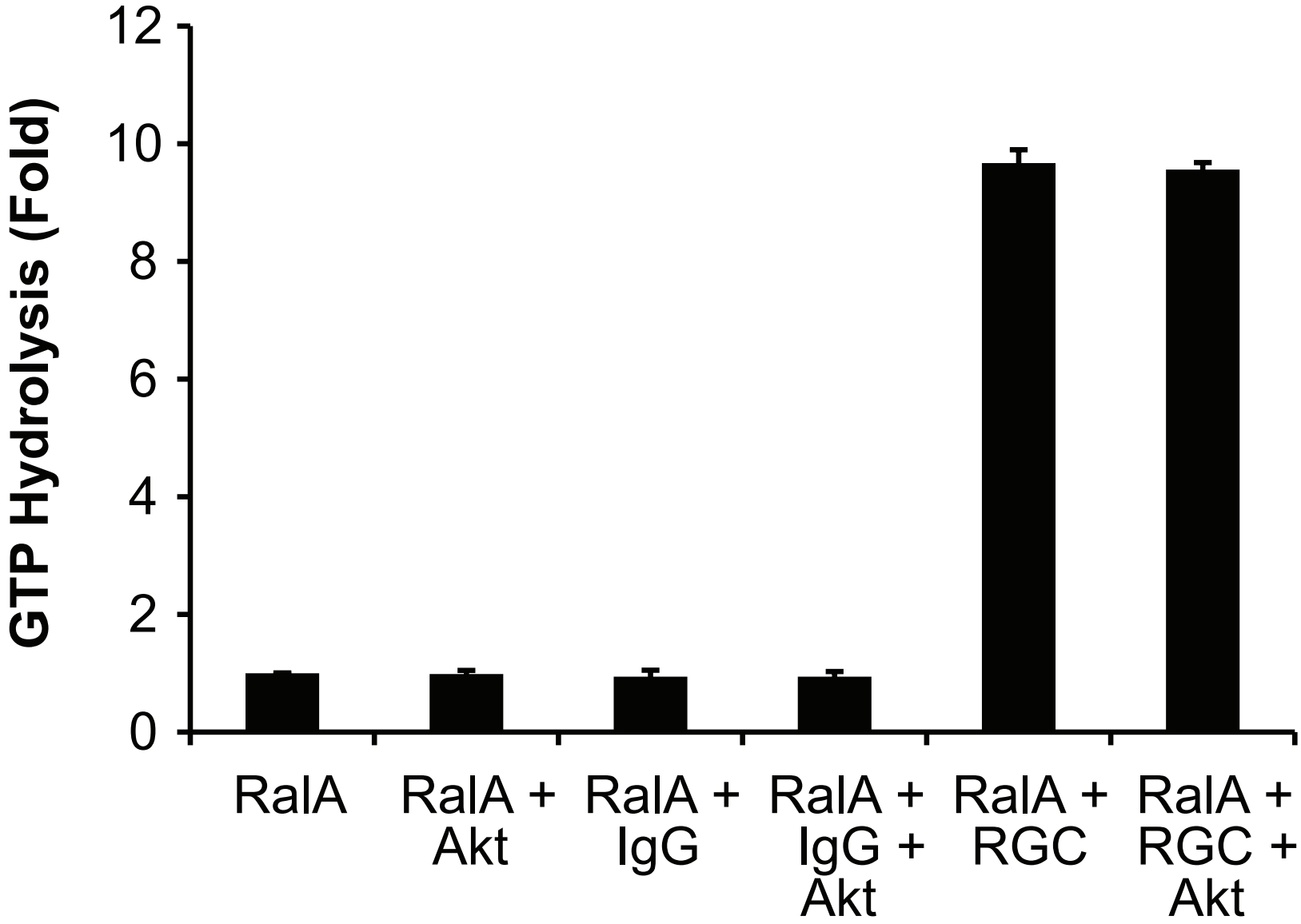

C
SVF
Adipocytes
IB: GARNL1
IB: RGC2
IB: RGC1
IB: Akt

Figure S5


GTP Hydrolysis (Fold)
12
10
8
6
4
2
0
RalA
RalA + Akt
RalA + IgG
RalA + IgG + Akt
RalA + RGC
RalA + RGC + Akt