SUPPLEMENTARY INFORMATION

DETAILED MATERIALS AND METHODS

Plasmids Constructs and protein expression—

Cloning of His-GIV-CT [1] and RNA interference-resistant GIV constructs [2] into p3XFLAG-CMV^{TM}-14 plasmid (GIV-FLAG) have been described previously. The following constructs were gifts from other investigators: HA-GLUT4-GFP from Samuel Cushman (NIH) [3]; Sec3-HA, Exo70-HA, Exo84-HA and Sec8-HA from Philippe Chavrier (Institut Curie, France) [4]; GST-Exo70 from Kun Ling (Mayo Clinic) [5]. Expression and purification of GST, GST-Exo70 and His-GIV CT using Bl21(DE3) (Invitrogen) were done as described previously [1,6,7]. Bacterial cultures were induced overnight at 25°C with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Pelleted bacteria from 1 L of culture were re-suspended in 10 ml GST-lysis buffer [25 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA, 20% (v:v) glycerol, 1% (v:v) Triton X-100, 2X protease inhibitor cocktail (Complete EDTA-free, Roche Diagnostics)] or His-lysis buffer [50 mM NaH_2PO_4 pH 7.4, 300 mM NaCl, 10 mM imidazole, 1% (v:v) Triton X-100, 2X protease inhibitor cocktail (Complete EDTA-free, Roche Diagnostics)] for GST or His-fused proteins, respectively. After sonication (3 x 30s), lysates were centrifuged at 12,000g at 4°C for 20 min. Solubilized proteins were affinity purified on glutathione-Sepharose 4B beads (GE Healthcare) or HisPur Cobalt Resin (Pierce). Proteins were eluted, dialyzed overnight against PBS and stored at -80 °C. GIV mutants were generated using specific primers (sequences available upon request) following the manufacturer´s instructions (QuickChange II, Stratagene).

Assay for measuring cell surface GLUT4—

The efficacy of GLUT4 translocation to the PM was analyzed by carrying out cell-surface immunofluorescence staining under non-permeabilizing conditions on cells expressing the

extensively characterized GLUT4 chimera, HA-GLUT4-GFP [3]. This chimera contains the HA tag in the first extracellular domain of GLUT4 and the GFP tag at the C-terminus of the receptor. Previous studies using this construct in HeLa cells [8] have demonstrated that dynamic translocation of this chimera to the PM after insulin stimulation closely resembles the endogenous transporter. Briefly, cells were transfected with HA-GLUT4-GFP, serum starved [using KRB buffer (Sigma-Aldrich) for 3 h] prior to stimulation with 100 nM insulin. Cells were fixed with 3% paraformaldehyde (PFA) in PBS and processed for immunofluorescence under non-permeabilizing conditions using as blocking buffer PBS with 3% BSA. The HA tag on the exterior of the cell surface was labeled with anti-HA (1:300; in red), whereas the C-terminal GFP tag allowed visualization of both cell surface and intracellular pool of GLUT4 (in green). Cells were imaged on a Leica SPE confocal microscope. All images were processed using ImageJ software and assembled into figure panels using Photoshop and Illustrator (Adobe). Approximately ~100 transfected cells (GFP positive) from randomly chosen fields were counted from three independent experiments. The percent of cells with GLUT4 translocated to the PM was determined by dividing the number of cells that stained positive for HA at the cell-surface/ the total number of GFP positive cells x 100.

Immunofluorescence and confocal microscopy—

Cells were fixed with 3% PFA in PBS for 25 min at room temperature, treated with 0.1 M glycine for 10 min, and subsequently blocked and permeabilized with PBS containing 3% BSA and 0.2% Triton X-100 (or only with 3% BSA in PBS buffer for surface labeling experiments where we used non-permeabilizing conditions) for 45 min at room temperature prior to antibody staining. Dilutions of antibodies are as follows: anti-phospho-GIV-Tyr1764 (1:200) (Ventana/Roche and Spring Biosciences); anti-HA (1:300) (Covance) and anti-GFP from Santa Cruz Biotechnologies; DAPI 1:1,000 (Molecular Probes); Secondary goat anti-rabbit (594) and goat anti-mouse (488) Alexa conjugated antibodies (1:500) from Molecular Probes. Coverslips

were mounted with ProLong Gold (Life Technologies). Cells were imaged on a Leica SPE confocal microscope using a 63x oil objective using 488, 561 and 405 laser lines for excitation. Images were scanned using a line-average of 3. All images were processed using ImageJ software and assembled into figure panels using Photoshop and Illustrator (Adobe).

Cell culture and transfection—

HeLa, Cos7 and L6 cells were cultured according to ATCC guidelines. Low passage (~3 to 8) L6 myotubes were cultured under non-differentiating conditions in α-MEM (Invitrogen) media supplemented with 10% FBS and differentiated into myotubes by switching to 2% FBS media for 6-7 days.

Transfection was carried out with GeneJuice (Novagen) for DNA plasmids or Oligofectamine (Invitrogen) for siRNA oligos according to manufacturer´s protocols. Silencer negative Control scrambled (Scr) siRNA used as control was purchased from Ambion and the previously validated [2] GIV siRNA sequence used was custom-ordered from Dharmacon.

Generation of stable cell lines—

HeLa cell lines stably expressing GIV-3xFLAG-WT, FA or SD mutants were extensively used in previous work [9]. GIV-3xFLAG expression was ~1-2 fold compared to the levels of endogenous GIV. In all experiments using stable cells, the endogenous GIV was depleted using GIV siRNA such that the cells exclusively expressed the desirable siRNA-resistant GIV mutant at near-physiologic levels.

Cell lysis and immunoblot analysis—

Whole cell lysates were prepared after washing cells with cold PBS prior to resuspending and boiling them in sample buffer. Cell lysates used as a source of proteins for pulldown assays or immunoprecipitation were prepared by breaking cells during passage through a 28-gauge

needle in the presence of lysis buffer [20 mM Hepes (pH 7.2), 5 mM Mg-acetate, 125 mM K-acetate, 0.4% Triton X-100, 1mM DTT, supplemented with sodium orthovanadate (500 μM), phosphatase (Sigma), and Protease (Roche) inhibitor cocktails] at 4ºC. Crude lysates were cleared of insoluble particles/debris by centrifugation at 10,000 x *g* for 10 minutes.

Protein samples for immunoblotting were run on SDS/PAGE gels and transferred onto PVDF membranes (Millipore). Membranes were blocked with PBS containing 5% non-fat milk (or with 5% BSA when probing for phosphorylated proteins) before incubation with primary antibodies. GIV was detected using rabbit anti-GIV coiled-coil (Millipore) (1:500) and GIV-CT (Santa Cruz Biotechnologies) (1:500). Other antibodies used in this work include mouse β-tubulin (1:1000) and GFP (1:500) from Santa Cruz Biotechnologies, His (1:1000) from Sigma, HA (1:500) from Covance, control IgG (Bio-Rad Laboratories) and phospho-Akt (Cell Signaling). Goat anti-rabbit and goat anti-mouse Alexa Fluor 680 or IRDye 800 $F(ab')_2$ secondary antibodies were purchased from Li-Cor Biosciences. Images were processed with ImageJ software (NIH) and assembled as figure panels using Photoshop and Illustrator software (Adobe).

GST pulldown assays and immunoprecipitations—

Purified GST-Exo70 or GST alone were immobilized on glutathione-Sepharose beads (GE Healthcare) for 1 h at room temperature as described before [6,7,10,11]. Cos7 lysates or His-GIV CT protein were added to each tube, and binding reactions were carried out for 4 h at 4ºC with constant tumbling in binding buffer [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.4% (v:v) Nonidet P-40, 10 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, protease inhibitor mixture]. Beads were washed (4x) with 1 mL of wash buffer [4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 0.1% (v:v) Tween 20, 10 mM $MgCl_2$, 5 mM EDTA, , 2 mM DTT] and bound complexes were eluted by boiling in Laemmli's sample buffer.

For immunoisolation of GSVs, homogenates of HeLa cells expressing HA-GLUT4-GFP (~2 mg of protein, no detergent) were incubated for 3 h at 4°C with 2 μg anti-HA and anti-GFP mAbs or pre-immune control mouse IgG. Protein G Sepharose beads (GE Healthcare) were added and incubated at 4°C for an additional 60 min. Beads were washed and bound immune complexes were eluted by boiling in Laemmli's sample buffer [6,7,12].

Proximity Ligation Assay (PLA)—

In situ interaction of endogenous active GIV (identified by anti-pTyr1764) or GIV-CT (T-13, Santa Cruz Biotechnologies) with HA-GLUT4-GFP or Exo70-HA was detected using the proximity ligation assay kit Duolink (Olink Biosciences). Cells were transfected and, 48 h later, cells were serum-deprived (3 h in KRB buffer) and stimulated with 100 nM insulin. Fixation, permeabilization and blocking were done as described for whole-cell immunofluorescence. PLA assay was performed according to manufacturer's recommendations. As negative control, cells incubated with only secondary antibodies were used.

Data Analysis and Statistics—

All experiments were repeated at least three times, and results were presented either as one representative experiment or as average ± S.D or S.E.M. Statistical significance was assessed with two-tailed Student’s t-test.

SUPPLEMENTARY FIGURE LEGENDS

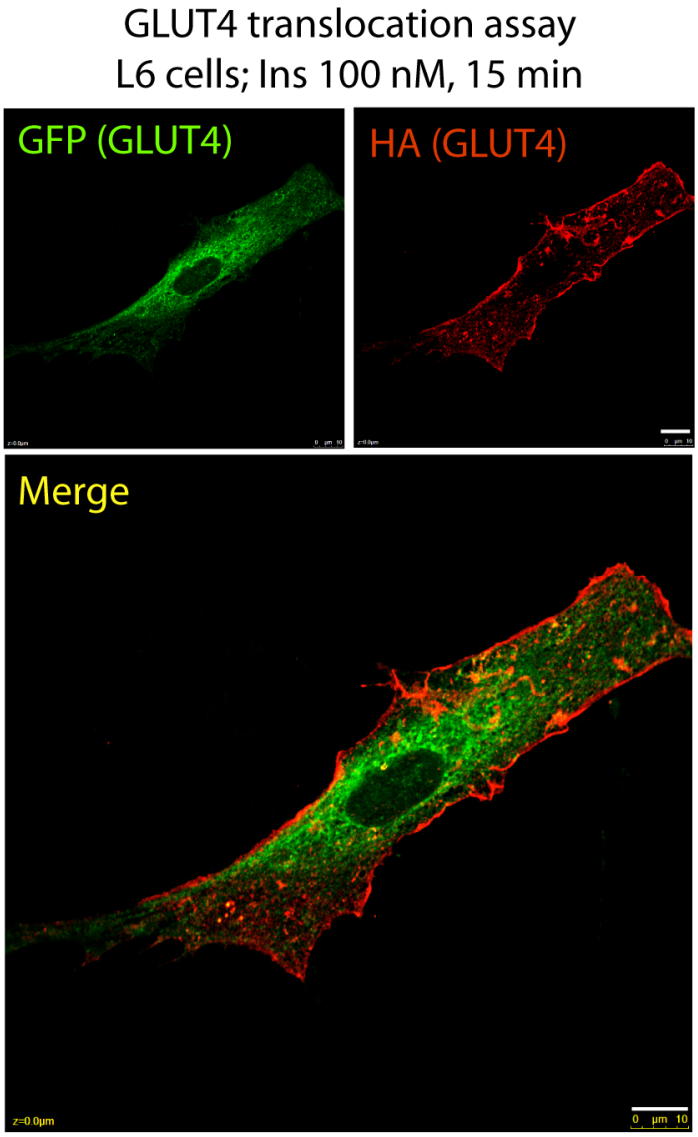

Fig S1: Analysis of GLUT4 translocation to the PM in response to insulin in L6 myotubes. L6 cells treated with control (Scr) or GIV siRNA were transfected with HA-GLUT4-GFP, serum-starved (3 h in KRB buffer) and stimulated with insulin. Subsequently, cells were fixed and stained for HA (red) and DAPI/DNA (blue). The intracellular pool of GLUT4 was detected by the GFP signal (green), whereas surface labelling of the exocytosed pool of GLUT4 was detected by staining the ectodomain using HA mAb. Cells were analyzed by confocal microscopy. Shown here is an image of a insulin-stimulated cell in which exocytosis was successful, as evidenced by the presence of anti-HA staining on the cell surface. Scale bar = 10 µm.

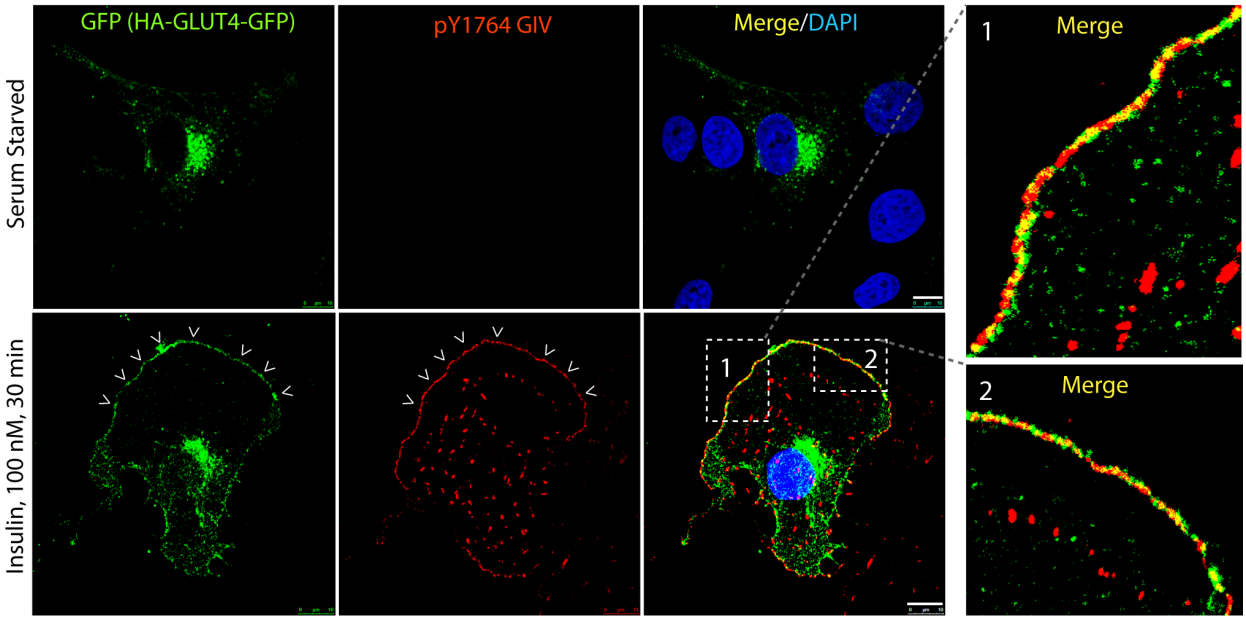

Fig S2: Tyrosine phosphorylated (active) GIV colocalizes with GCVs at the cell periphery after insulin stimulation. Serum starved Cos7 cells expressing HA-GLUT4-GFP were stimulated with insulin prior to fixation. Fixed cells were stained for pY1764-GIV (red) and DAPI/DNA (blue) and analyzed by confocal microscopy. GLUT4 was detected by GFP (green). Individual green and red panels show GLUT4-containing puncta and tyrosine phosphorylated GIV at the cell periphery (arrowheads). Yellow pixels in the magnified merged panels show that GLUT4 containing puncta and GIV colocalize partially at the PM. Scale bar = 10 μm.

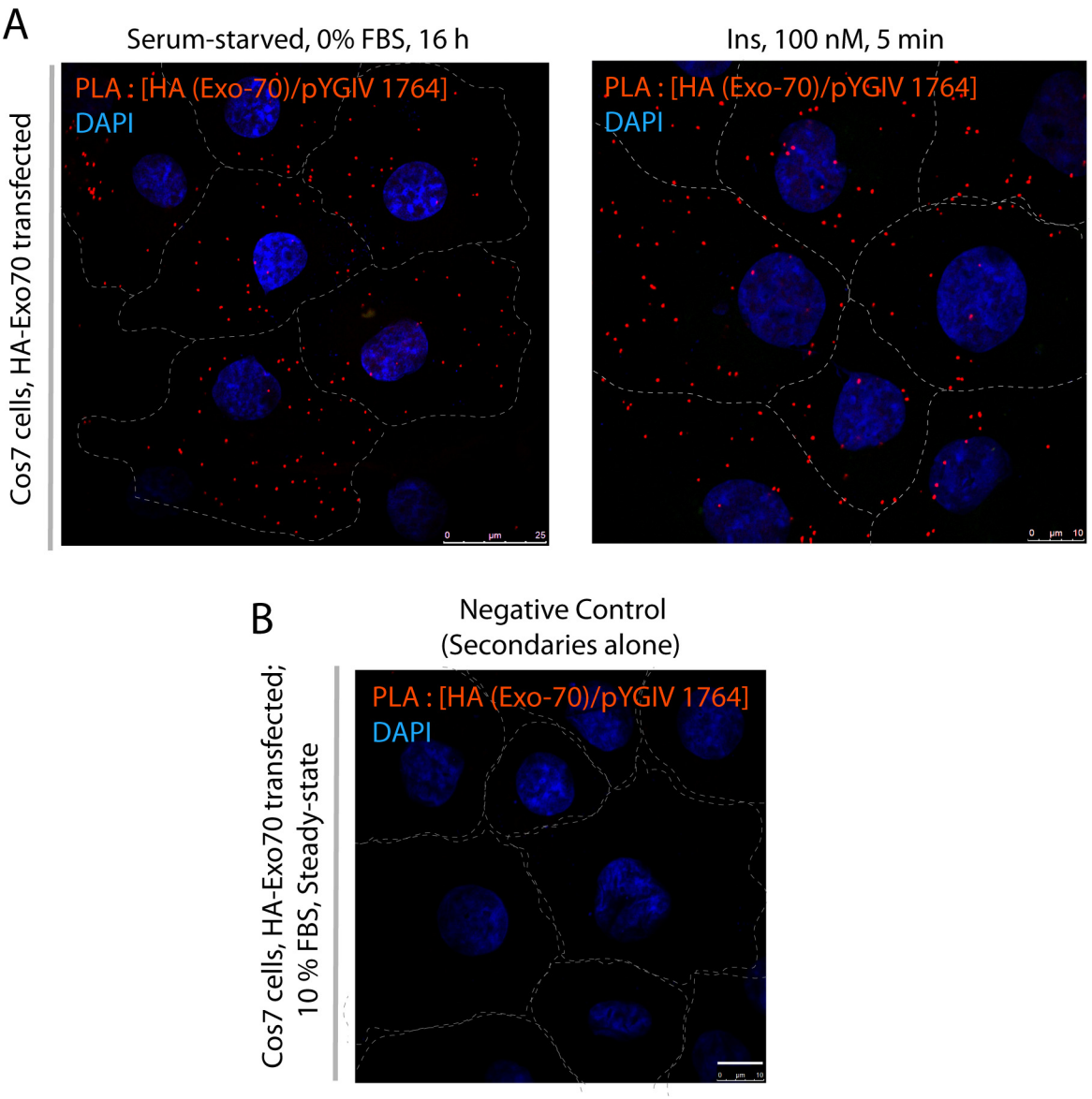

Figure S3: Active GIV interacts constitutively with Exo70. (A-B) Serum starved Cos7 cells expressing Exo70-HA were stimulated with insulin prior to fixation, and subsequently analyzed for interaction between active GIV and Exo-70 by *in situ* PLA using rabbit pY1764-GIV and mouse anti-HA **(A)**. Scale bar = 25 μm. As negative control **(B)**, cells were incubated with PLA secondary antibodies alone. Red dots = interaction. Interactions were noted as equal in both starved and insulin stimulated cells, whereas no interaction was seen in the negative control. Scale bar = 10 μm.

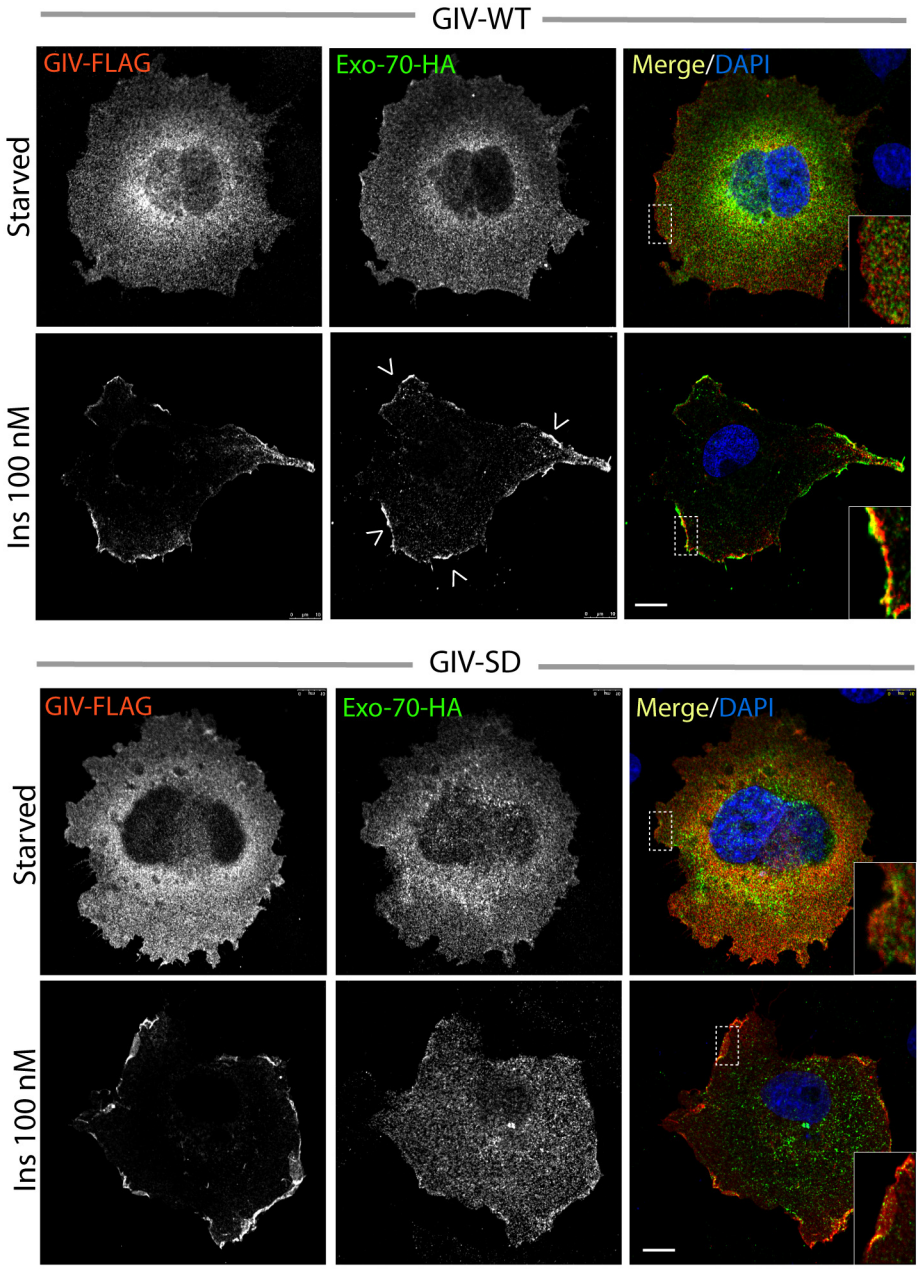

Figure S4: Phosphoinhibition of GIV's GEF at S1689 inhibits PM localization of Exo-70 after insulin stimulation. Cos7 cells coexpressing Exo-70-HA and either GIV-WT-FLAG or GIV-SD-FLAG were serum starved and insulin-stimulated and then stained with HA (green; individual panel shown in grayscale), FLAG (red; individual panel shown in grayscale) and DNA/DAPI (blue). Arrowheads = PM. Bar = 10 μm.

BIBLIOGRAPHY

[1] M. Garcia-Marcos, P. Ghosh, M.G. Farquhar, GIV is a nonreceptor GEF for G alpha i with a unique motif that regulates Akt signaling, Proc Natl Acad Sci U S A 106 (2009) 3178-3183.

[2] A. Enomoto, H. Murakami, N. Asai, N. Morone, T. Watanabe, K. Kawai, Y. Murakumo, J. Usukura, K. Kaibuchi, M. Takahashi, Akt/PKB regulates actin organization and cell motility via Girdin/APE, Dev Cell 9 (2005) 389-402.

[3] K. Dawson, A. Aviles-Hernandez, S.W. Cushman, D. Malide, Insulin-regulated trafficking of dual-labeled glucose transporter 4 in primary rat adipose cells, Biochem Biophys Res Commun 287 (2001) 445-454.

[4] M. Sakurai-Yageta, C. Recchi, G. Le Dez, J.B. Sibarita, L. Daviet, J. Camonis, C. D'Souza-Schorey, P. Chavrier, The interaction of IQGAP1 with the exocyst complex is required for tumor cell invasion downstream of Cdc42 and RhoA, J Cell Biol 181 (2008) 985-998.

[5] X. Xiong, Q. Xu, Y. Huang, R.D. Singh, R. Anderson, E. Leof, J. Hu, K. Ling, An association between type Igamma PI4P 5-kinase and Exo70 directs E-cadherin clustering and epithelial polarization, Mol Biol Cell 23 (2012) 87-98.

[6] P. Ghosh, A.O. Beas, S.J. Bornheimer, M. Garcia-Marcos, E.P. Forry, C. Johannson, J. Ear, B.H. Jung, B. Cabrera, J.M. Carethers, M.G. Farquhar, A G{alpha}i-GIV molecular complex binds epidermal growth factor receptor and determines whether cells migrate or proliferate, Mol Biol Cell 21 (2010) 2338-2354.

[7] P. Ghosh, M. Garcia-Marcos, S.J. Bornheimer, M.G. Farquhar, Activation of Galphai3 triggers cell migration via regulation of GIV, J Cell Biol 182 (2008) 381-393.

[8] Y. Haga, K. Ishii, T. Suzuki, N-glycosylation is critical for the stability and intracellular trafficking of glucose transporter GLUT4, J Biol Chem 286 (2011) 31320-31327.

[9] I. Lopez-Sanchez, M. Garcia-Marcos, Y. Mittal, N. Aznar, M.G. Farquhar, P. Ghosh, Protein kinase C-theta (PKCtheta) phosphorylates and inhibits the guanine exchange factor, GIV/Girdin, Proc Natl Acad Sci U S A 110 (2013) 5510-5515.

[10] M. Garcia-Marcos, P.S. Kietrsunthorn, Y. Pavlova, M.A. Adia, P. Ghosh, M.G. Farquhar, Functional characterization of the guanine nucleotide exchange factor (GEF) motif of GIV protein reveals a threshold effect in signaling, Proc Natl Acad Sci U S A 109 (2012) 1961-1966.

[11] C. Lin, J. Ear, Y. Pavlova, Y. Mittal, I. Kufareva, M. Ghassemian, R. Abagyan, M. Garcia-Marcos, P. Ghosh, Tyrosine phosphorylation of the Galpha-interacting protein GIV promotes activation of phosphoinositide 3-kinase during cell migration, Sci Signal 4 (2011) ra64.

[12] M. Garcia-Marcos, J. Ear, M.G. Farquhar, P. Ghosh, A GDI (AGS3) and a GEF (GIV) regulate autophagy by balancing G protein activity and growth factor signals, Mol Biol Cell 22 (2011) 673-686.