SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Confirmation of efficient depletion of Gi3 and GIV by siRNA in HeLa cell lines. (A) Equal aliquots of whole cell lysates of HeLa cells treated with Scramble (Scr, lane 1), Gi3 (lane 2) or GIV (lane 3) siRNA as in Figure 1A were analyzed for GIV, Gi3, Gβγ, and tubulin by immunoblotting (IB). Quantification by LiCOR infrared Odyssey imager confirmed efficient depletion of Gi3 (~ 85-95%) and GIV (~ 80-90%), and levels of Gβγ do not change significantly when GIV or Gi3 are depleted. (B) Equal aliquots of whole cell lysates of control HeLa cells and HeLa cells stably expressing siRNA-resistant (siRest) GIV-WT or GIV-F1685A plasmids were separated by SDS-PAGE and analyzed for GIV and tubulin by immunoblotting (IB). While endogenous GIV was effectively depleted in controls (compare lanes 1 and 2), siRNA-resistant GIV-WT and GIV-F1685A proteins were stably expressed (lanes 3 and 4).

Figure S2. Insulin reverses autophagy in GIV-WT, but not in the GEF deficient GIV-FA cells, as determined by staining for endogenous LC3. Control HeLa cells, or HeLa cells stably expressing either siRNA-resistant wild-type GIV (GIV-WT) or GEF-deficient GIV (GIV-FA) (Figure S1B), were treated with scrambled (Scr) or GIV siRNA as indicated. Cells were starved for 8 h in 0.2% FBS, prior to stimulation with 100 nM insulin for 1 h (as in 1A), fixed, stained for LC3 (red) and the nucleus/DAPI (blue), and analyzed by confocal microscopy. Scr-treated controls showed multiple LC3-positive vesicles when starved (a), and recovery of phenotype with disappearance of LC3-positive vesicles upon insulin treatment (b). GIV-WT cells showed identical staining as controls (c, d), whereas GIV-FA cells showed persistence of LC3-positive vesicles despite insulin treatment (f). Bar = 10 µM.

Figure S3. Fusion of autophagosomes with lysosomes takes place in GIV-FA cells. HeLa GIV-FA cells expressing GFP-LC3 were either maintained in 10% serum (a, b) or starved (c, d) in the presence of leupeptin and pepstatin for 12 h (to inhibit lysosomal proteases), fixed, processed for semi-thin cryosections, and stained for GFP-LC3 (GFP mAb, green) and the lysosomal marker, Cathepsin D (affinity purified rabbit IgG, red). In the presence of leupeptin and degradation of the autophagolysosomal contents is blocked (Gohla et al., 2007). Under both conditions, GFP-LC3 and Cathepsin D show striking colocalization (yellow pixels, arrowheads in b, d), indicating efficient fusion of GFP-LC3-labeled autophagosomes with Cathepsin D-labeled lysosomes. Similar colocalizations were also seen in control HeLa and GIV-WT cells (data not shown). These results demonstrate that LC3-positive nascent autophagosomes fuse successfully with lysosomes in GIV-FA cells, indicating that the GFP-LC3-positive autophagosomes efficiently mature into autophagosomes in these cells. Bar = 5 µM.

Figure S4. Gi3 preferentially coimmunoprecipitates with AGS3 in starved cells and with GIV upon EGF stimulation. Cos7 cells transiently transfected with FLAG-tagged Gi3 (Gi3-FLAG) or vector control were starved and stimulated with 50 nM EGF for 15 min prior to lysis. Equal aliquots of lysates (left panels) were incubated with anti-FLAG mAb. Immunoprecipitated complexes (right panel) were analyzed for GIV, AGS3 and FLAG (Gi3) by immunoblotting (IB). Gi3-bound immune complexes were simultaneously depleted of AGS3 and enriched in GIV upon insulin treatment.
SUPPLEMENTAL MATERIALS AND METHODS

Reagents and Antibodies
Unless otherwise indicated all reagents were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO). Cell culture media were purchased from Invitrogen (Carlsbad, CA). Epidermal growth factor (EGF) and insulin were obtained from Invitrogen and Novagen, respectively. Silencer Negative Control scramble (Scr) siRNA and Gαi3 siRNA (Ghosh et al., 2008) were purchased from Ambion and Santa Cruz, respectively, whereas GIV siRNA (Ghosh et al., 2008; Garcia-Marcos et al., 2009) was custom ordered from Dharmacon. GIV was detected by immunoblotting using anti-GIV coiled-coil serum (GIV-ccAb) raised against the coiled coil domain of GIV (Le-Niculescu et al., 2005), and affinity purified anti-GIV/Girdin C-terminus (GIV-CTAb) raised against the last 19 aa of GIV’s C-terminus (IBL America, Minnesota). AGS3 was detected using an affinity purified anti-AGS3 IgG raised against the last 14 aa (KGPDPQKQSSPQPSSGAS) at the C terminus of AGS3 (De Vries et al., 2000). Affinity purified pAbs against LC3 and cathepsin D were generous gifts from Takashi Ueno (Juntendo University, Tokyo, Japan) and Stuart Kornfeld (Washington University), respectively. Other antibodies used in this work include rabbit pAbs against Gαi3 (M-14) and pan-Gβ (Santa Cruz Biotechnology, CA), phospho-S6K, phospho-Akt Ser 473, phospho-S6Ribosomal protein, and phospho-ERK 1/2 (Cell Signaling), and mouse mAbs against GFP (Living Colors, Invitrogen), HA (Covance); FLAG, tubulin and polyhistidine (Sigma), Akt (BD Biosciences), ERK 1/2 (Cell signaling Technologies), which were obtained commercially. Anti-mouse and anti-rabbit Alexa-594- and Alexa-488-coupled goat secondary antibodies for immunofluorescence were purchased from Invitrogen. Goat anti-rabbit and goat anti-mouse Alexa Fluor 680 or IRDye 800 F(ab')2 for immunoblotting were from Li-Cor Biosciences (Lincoln, NE). Control mouse and rabbit IgGs for immunoprecipitation were purchased from BioRad (Hercules, CA) and Sigma (St. Louis, MO), respectively.

Plasmid Constructs and Mutagenesis
GST-LC3 and GFP-LC3 plasmids were generous gifts from Karla Kirkegaard (Stanford University School of Medicine) and Catherine Denicourt (University of Texas Health Science Center at Houston), respectively. Full length AGS3 (650 aa) cDNA was obtained previously (De Vries et al., 2000) by reverse transcription (RT)-PCR on rat brain cDNA based on the reported sequence (GenBank no. AF107723) and cloned into pcDNA3 fused to an N-terminal HA tag as described previously (Pattingre et al., 2003). A cDNA fragment encoding the GoLoco domains of AGS3 (aa 424-650) was subcloned into pET28a vector (Novagen) using Ndel and Eco RI sites. The GST-AGS3 (aa 465-650) plasmid was a generous gift from Stephen Lanier (University of South Carolina). Cloning of Gαi3 and GIV-CTs (aa 1660-1870) into pGEX-4T-1 or pET28b were described previously (Garcia-Marcos et al., 2009). To transiently express C-terminal FLAG-tagged Gai3 in COS-7 cells, Gai3 was cloned into p3XFLAG-CMV/TM-14 expression vector using BamH I and Hind III restriction enzymes (Garcia-Marcos et al., 2010). GIV mutants (Garcia-Marcos et al., 2009) were generated using specific primers (sequences available upon request) following the manufacturer's instructions (QickChange II, Stratagene, San Diego, CA). RNAi-resistant GIV was generated by silent mutations as described. All constructs were checked by DNA sequencing.

Cell Culture, Transfection and Lysis
Unless mentioned otherwise, all cell lines used in this work were cultured according to ATCC guidelines. Transfection was carried out using Genejuice (Novagen) for DNA plasmids or Oligofectamine (Invitrogen) for siRNA oligos following the manufacturers' protocols, and stable cell lines were selected as mentioned previously (Garcia-Marcos et al., 2009; Ghosh et al., 2010) using the neomycin analogue, G418 (Cellgro). Transfections to transiently overexpress proteins using AGS3-HA, GFP-LC3, and Gai3-FLAG plasmids or protein silencing using siRNA GIV or Gai3 were carried out.
exactly as described. In the case of GFP-LC3, plasmid cDNA levels were carefully titrated to minimal levels (0.15 µg and 1 µg of plasmid cDNA / well of 6-well dish or 10 cm dish, respectively) that achieved adequate expression and showed dynamic changes in pattern in the presence or absence of growth factors, while avoiding excessive levels. HeLa cell lines stably expressing GIV-WT (HeLa-GIV-WT) or GIV-F1685A mutant (HeLa-GIV-FA) were generated and maintained as previously described and maintained in the presence of G418 (500 µg/ml). Clones were chosen for each construct that had relatively low expression levels of GIV (~3 times higher than endogenous levels). For each construct, two separate clones were investigated, and similar results were obtained.

For insulin/EGF stimulation assays involving serum starvation, the serum concentration was reduced to 0.2% for 12 h, and if indicated, cells were then stimulated with 50 nM EGF or 100 nM Insulin. Whole cell lysates were prepared after washing cells with cold PBS by resuspending cells in sample buffer and boiling immediately.

Lysates used as a source of proteins in immunoprecipitation or pull-down assays were prepared by resuspending cells in lysis buffer [20 mM HEPES, pH 7.2, 5 mM Mg-acetate, 125 mM K-acetate, 0.4% Triton X-100, 1 mM DTT, supplemented with sodium orthovanadate (100 µM), phosphatase (Sigma) and protease (Roche) inhibitor cocktails], after which they were passed through a 30G needle at 4 °C, and cleared (10-14,000g for 10 min) before use in subsequent experiments.

Statistical Analysis
Each experiment presented in the figures is representative of at least three independent experiments. Statistical significance (p value) between various conditions was assessed with the Student’s t-test. All graphical data presented was prepared using GraphPad Software, Inc., San Diego, CA.
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Figure S1
Figure S2
Figure S4