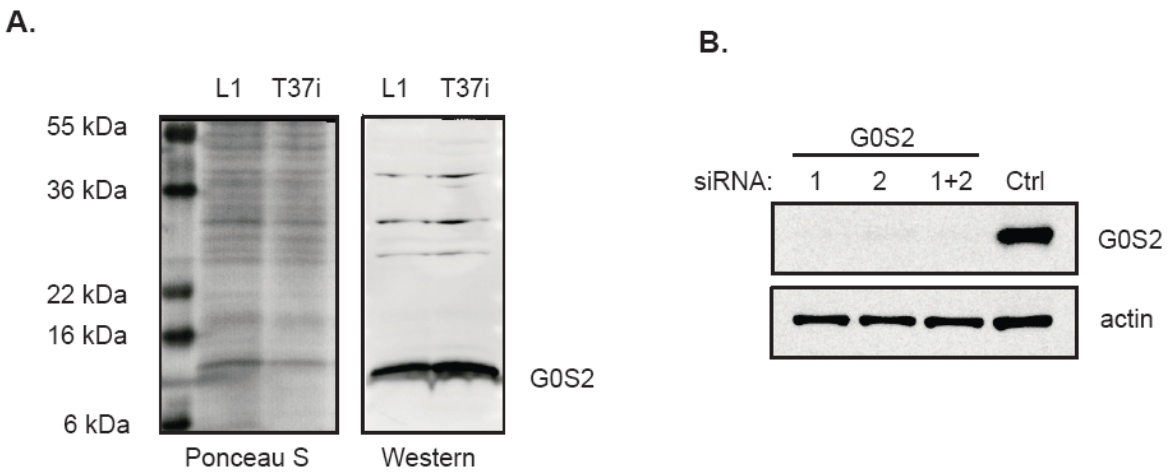
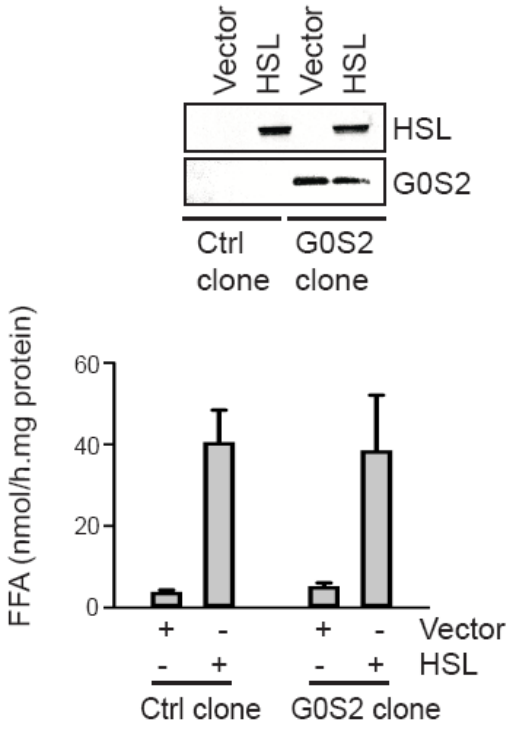
Figure S1. G0S2 antibody specifically recognizes endogenous GOS2 in adipocytes.



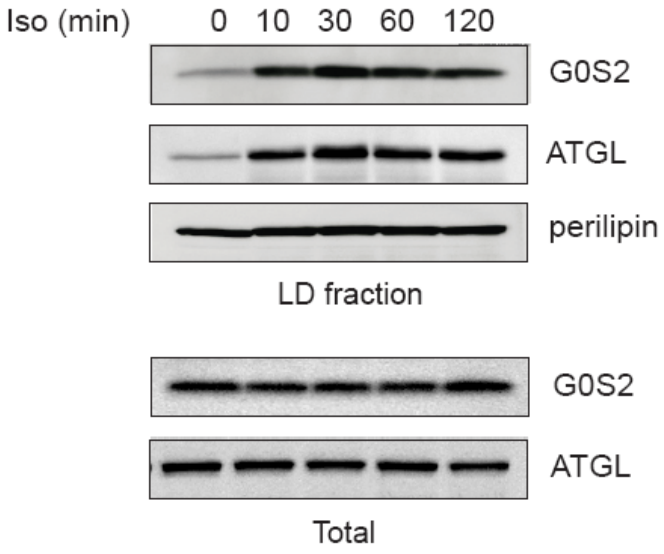
A. Lysates from 3T3-L1 and T37i adipocytes were analyzed for G0S2 protein by immunoblotting (20 μg of protein/lane) using an affinity-purified polyclonal antibody raised against the carboxyl-terminal (amino acids 43-103) domain of murine G0S2. A major protein band of 11 kDa was detected that matched the predicated molecular weight of G0S2. The Ponceau S staining of the nitrocellulose membrane is shown as loading control. **B.** siRNA-mediated knockdown in 3T3-L1 adipocytes was performed by electroporating 3T3-L1 adipocytes with either control siRNA or G0S2-specific siRNA (oligo 1, 2 or 1+2). Expression of G0S2 protein was analyzed 3 days later by immunoblotting with G0S2 antibody, using β-actin as a loading control. The specific band recognized by G0S2 antibody was not observed when 3T3-L1 cells were pretreated with two siRNA oligos directed against separate regions of G0S2 sequence, demonstrating the specificity of the antibody.

Figure S2. G0S2 does not affect *in vitro* TAG hydrolase activity of HSL.



HeLa cells from control clone or G0S2-expressing clone were transfected with vector alone or HSL. 24 h after transfection, protein expression was analyzed by immunoblotting with anti-HSL and anti-G0S2 antibodies. The TAG hydrolase activity in the cell extracts was measured using ^{3}H-labeled triolein as substrate. The activity was normalized with the total protein levels of the cell extracts.

Figure S3. Lipid droplet translocation of ATGL and G0S2 in adipocytes.



Lipid droplets (LD) were isolated from 3T3-L1 adipocytes pretreated with 1 μM isoproterenol/0.25 mM IBMX for varying length of time. Immunoblotting analysis using perilipin as control showed that G0S2 and ATGL underwent rapid and sustained relocalization to lipid droplets in response to isoproterenol stimulation. During the 2 h time span, total levels of intracellular ATGL and G0S2 protein remained unchanged.

Supplemental Experimental Procedures

Antibodies and reagents

The ATGL blocking peptide (cat# 1006) and the rabbit polyclonal antibodies against ATGL (cat# 2439 & cat#2138), BiP (cat# 3177) and HSL (cat# 4107) were from Cell Signaling. Monoclonal β-actin antibody (cat# A1978) and β-tubulin antibody (cat# T4026) were obtained from Sigma-Aldrich. Monoclonal anti-CGI-58 antibody (cat# sc-100468) was purchase from Santa Cruz Biotech. Affinity-purified G0S2 rabbit polyclonal antibody was generated against recombinant GST fusion protein containing the C-terminal region of murine G0S2 (residues 43-103) by Proteintech Group, Inc. The goat polyclonal antibody against perilipin A (cat# ab616682) and rabbit polyclonal antibody against UCP1 (cat# ab10983) were purchased from Abcam Inc. The aP2 polyclonal antibody was kindly provided by Dr. David A. Bernlohr. Horseradish peroxidase-linked secondary antibodies were from Pierce Chemical Co. The protease inhibitor mini tablets (cat# 11 836 170 001) were obtained from Roche Diagnostics. TNT® SP6 high-yield protein expression system (cat# L3261) was from Promega Co. Triglyceride assay kit (cat# TG-1-NC) and lipolysis assay kit (cat# LIP-3-NC-L1) were purchased from Zenbio. ^{3}H-labeled triolein was from Perkin Elmer Co. QuickChange site-directed mutagenesis kit (cat# 500518) was purchased from Stratagene. Vectashield mounting medium was purchased from Vector Laboratories. The Alexa Fluor secondary antibodies, Lipofectamine 2000 transfection reagent, hygromycin B and reagents for tissue culture were obtained from Invitrogen. GW501516 was purchased from Cayman Chemical. Rosiglitazone was obtained from Gateway Chemical Technology, LLC. TNFα

and 3,3′,5′-triiodo-L-thyronine (T3), insulin, dexmethasone, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich.

Animal tissue extraction

100 mg of mouse tissues collected from 24-week-old C57BL6 mice were placed in tissue grinder vessel containing 0.4 ml homogenization/lysis buffer (50 mM Tris pH7.4; 150 mM NaCl; 1% Triton X-100, 1% NP-40, 0.1% SDS, and protease inhibitors at 1 tablet per 7 ml of buffer). Homogenization was conducted by using a drill press (Delta Shopmaster, model# DP350) with 8 stokes for adipose tissue or 12 strokes for other tissues. Completely homogenized tissues were incubated on ice for 20 min and centrifuged at 1000 x g at 4ºC for 10 min. The clarified lysates were mixed with 2× SDS sample buffer, and the solubilized proteins were detected using immunoblotting analysis.

Cell culture

HeLa cells (ATCC) were cultured in DMEM containing 10% fetal bovine serum (FBS). Mouse 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% newborn calf serum, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. Differentiation to adipocytes was induced by treatment of postconfluent cells with 10% FBS, 1 μg/mL insulin, 1 μM dexamethasone (DEX), and 0.5 mM isobutyl-1-methylzanthine (IBMX). The differentiation medium was withdrawn 3 days later and replaced with medium supplemented with 10% FBS and 1 μg/mL insulin. After 2 days in insulin containing medium, the cells were then cultured in DMEM containing 10% FBS. T37i brown preadipocytes were cultured and differentiated as described previously

(Zennaro et al., 1998). Briefly, T37i preadipocytes were cultured in DMEM-Ham's F12 medium supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 20 mM HEPES. Differentiation into brown adipocytes was achieved under standard conditions by incubating subconfluent undifferentiated preadipocytes with 2 nM triiodothyronine (T3) and 20 nM insulin for ≥ 5 days.

RNA extraction, PCR cloning of cDNA and site-directed mutagenesis

Total RNA was prepared from mouse 3T3-L1 adipocytes using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instruction. cDNA was prepared from mRNA using SuperScript Reverse Transcriptase protocol (Invitrogen). The sequences containing the complete open reading frame of mouse ATGL, HSL, G0S2 were amplified by PCR using ultra Pfu DNA Polymerase Mix (Stratagene). The primers designed to create restriction sites for subsequent cloning strategies are as follows: G0S2 forward, 5'-CGC GGA TCC ATG GAA AGT GTG CAG GAG CTG ATC C-3'; G0S2 reverse, 5'-CCG CTC GAG TTA AGA GGC GTG CTG CCG GAG GGA C-3'; G0S2 reverse (for cloning in pKF vector), 5'-CCG CTC GAG AGA GGC GTG CTG CCG GAG GGA C-3'; ATGL forward, 5'-GCA GAT CTA TGT TCA CGA GGG AGA CCA AGT GGA-3'; ATGL reverse, 5'-CGC TCG AGT CAG CAA GGC GGG AGG CCA GGT-3'; HSL forward, 5'-GCG GAT CCA TGG ATT TAC GCA CGA TGA CAC AG-3'; HSL reverse, 5'-GTA CTC GAG TCA GTT CAG TGG TGC AGC AGG-3'; CGI58 forward, 5'-GCG GAT CCA AAG CGA TGG CGG CGG AGG AGG-3'; CGI58 reverse, 5'-CGC TCG AGT CAG TCT ACT GTG TGG CAG ATC-3'.

The PCR products containing G0S2 and HSL cDNA were cleaved by BamHI / XhoI, and the PCR product containing ATGL was digested by Bgl II / XhoI. The digested products were purified and ligated to BamHI (5′) and XhoI (3′) sites of the eukaryotic expression vector pRK7. To generate a construct containing G0S2 fused at the C-terminus with a FLAG epitope tag (G0S2-FLAG), digested G0S2 cDNA was ligated into pKF vector (Liu et al., 2005). Deletion mutations were generated by using the QuickChange site-directed mutagenesis kit according to manufacturer's guidelines. pRK-ATGL and pRF-G0S2 constructs were used as templates for mutagenesis reactions. The primers designed to create internal deletions are as follows: G0S2ΔHD forward, 5'-AGG GAA GCT AGT GAA GCT AGT TGA GAC GGT GTG CAG C-3'; G0S2ΔHD reverse, 5'-GCT GCA CAC CGT CTC AAC TAG CTT CAC TAG CTT CCC T-3'; ATGLΔPT forward, 5'-CGA GGG AGA CCA AGT GGA ACC CTA CCC TCC AAG GGG TGC G-3'; ATGLΔPT reverse, 5'-CGC ACC CCT TGG AGG GTA GGG TTC CAC TTG GTC TCC CTC G-3'; ATGLΔHD forward, 5'-GCC CTG CTG GAG GCC TGC AGA CTG TCT GAG CAG GTG G-3'; ATGLΔHD reverse, 5'-CCA CCT GCT CAG ACA GTC TGC AGG CCT CCA GCA GGG C-3'.

Transient and stable transfection of HeLa cells

Transient transfection was performed using Lipofectamine 2000 reagent according to the manufacturer's instructions. For immunofluorescence studies, 0.5 μg of each DNA construct was transfected into cells cultured on coverslips at low density in 6-well dishes. Cells were fixed 16-18 h post transfection. For immunoblotting and immunoprecipitation analysis, 1.0 μg of each DNA was used in transfection of subconfluent cells cultured in

60-mm dishes. Cells were lysed 16-18 h post transfection. To establish stable cell clones, HeLa cells plated in 10-cm dishes were transiently transfected with 10 μg of pRK-G0S2 or pRK vector alone along with 1 μg of pcDNA3 vector carrying a hygromycin resistance gene. 16 h after transfection, cells were trypsinized and were individually plated in 96-well dishes. Stable expression was selected by culturing the transfected cells in medium containing 50 mg/ml of hygromycin B. After approximately 3 weeks, colonies were expanded and expression of G0S2 was subsequently verified by immunoblotting analysis.

Cell lysis, immunoblotting and immunoprecipitation

For immunoblotting, HeLa, 3T3-L1 and T37i cells were washed twice with ice-cold PBS, and were lysed at 4°C with a buffer containing 50mM Tris-HCl (pH 8.0), 135mM NaCl, 10 mM NaF, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1.0 mM EDTA, 5% glycerol and protease inhibitors (1 tablet per 7 ml of buffer). The lysates were clarified by centrifugation at 10,000 x g for 10 min and then mixed with equal volume of 2× SDS sample buffer. The solubilized proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Individual proteins were blotted with primary antibodies at appropriate dilutions. Peroxide-conjugated secondary antibodies were incubated with the membrane at a dilution of (1:5000). The signals were then visualized by enhanced chemiluminescence (ECL Reagents, GE Healthcare). For immunoprecipitation, cells were lysed in a buffer containing 50mM Tris-HCl (pH 8.0), 135mM NaCl, 10 mM NaF, 1% NP-40, 1.0 mM EDTA, 5% glycerol and protease inhibitors. The clarified lysate were allowed to mix with appropriate amount of primary antibodies for 1 h at 4ºC. For immunoprecipitation of ATGL and peptide competition, 2.5 μg of rabbit monoclonal

ATGL antibody (cat# 2439) per mL of lysate was used along with or without 10 μg of ATGL blocking peptide. The protein A/G agarose beads were then added and incubation continued for 2 h. Following extensive washes with the same lysis buffer, the agarose beads were mixed with 1× SDS sample buffer and the proteins were detected using immunoblotting analysis.

Immunofluorescence staining and confocal microscopy

HeLa cells and adipocytes were maintained at proper densities on glass cover slips placed in 6-well dishes. Following the fixation with 3% paraformaldehyde in PBS for 30 min, cells were quenched with 100 mM glycine in PBS for 20 min and then blocked with 1% BSA/ 0.01% Saponin in PBS for 1h. The cells were then exposed to primary antibodies for 2 h at room temperature. Following three washes with PBS, the cells were treated for 1 h with Alexa Fluor secondary antibodies diluted to 2 μg/mL in blocking solution. To visualize lipid droplets, BODIPY 493/503 dye was added at a final concentration of 0.3 μg/mL during the incubation with secondary antibodies. Samples were mounted on glass slides with Vectashield mounting medium and examined under a Leica SP5 inverted confocal microscope. For lipid droplet size quantification, confocal images were analyzed using the Leica LAS AF software.

In vitro transcription-and-translation expression and biochemical assay

In vitro transcription/translation was carried out by using TNT® SP6 High-Yield Protein Expression System according to the manufacture's instruction. Specifically, 10 μg vector DNA per reaction was used to produce ATGL, G0S2 and CGI-58 for TAG hydrolase

assays. For coimmunoprecipitation experiments, 4 μg of pRK-ATGL was included in each reaction along with 2 μg of either pKF-G0S2 or pKF vector alone.

References

Liu, J., Deyoung, S.M., Zhang, M., Dold, L.H., and Saltiel, A.R. (2005). The stomatin/prohibitin/flotillin/HflK/C domain of flotillin-1 contains distinct sequences that direct plasma membrane localization and protein interactions in 3T3-L1 adipocytes. J Biol Chem 280, 16125-16134.

Zennaro, M.C., Le Menuet, D., Viengchareun, S., Walker, F., Ricquier, D., and Lombes, M. (1998). Hibernoma development in transgenic mice identifies brown adipose tissue as a novel target of aldosterone action. J Clin Invest 101, 1254-1260.