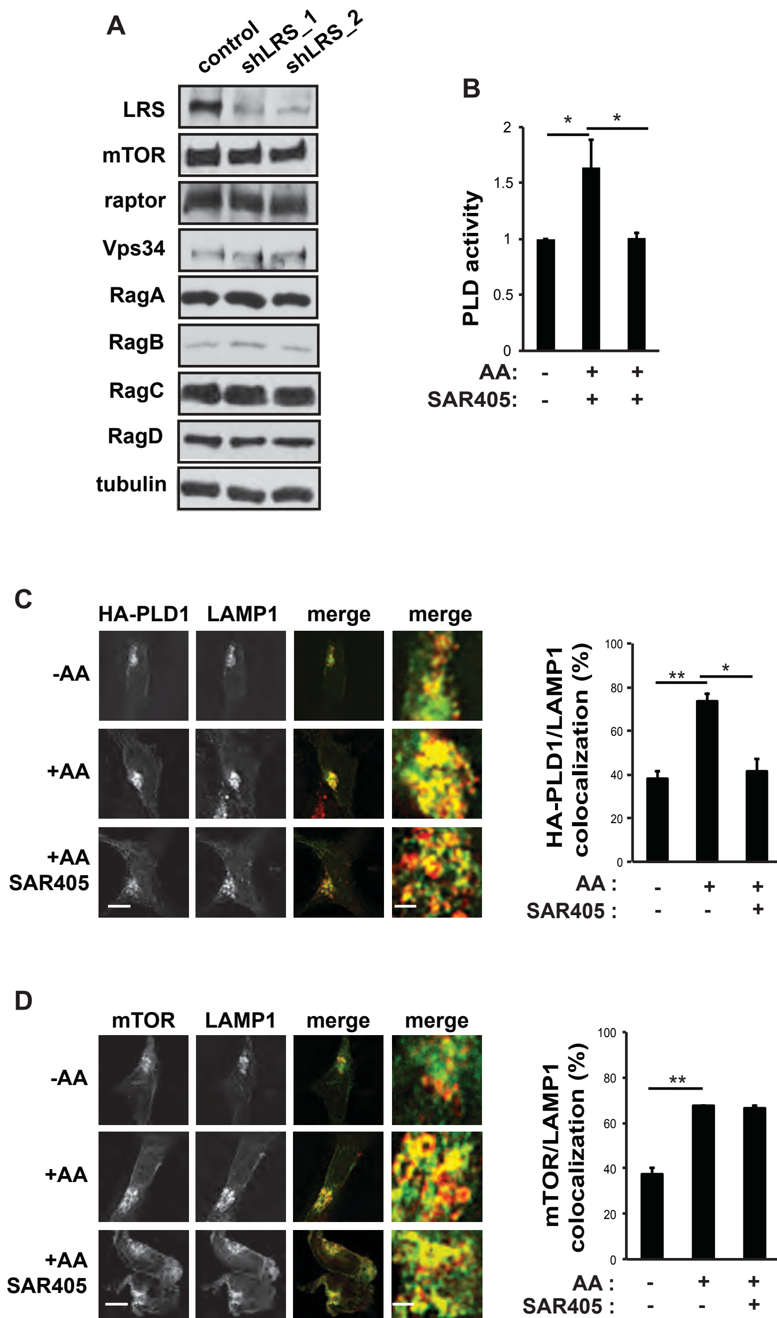

Figure S1 – related to Figure 1. SAR405 inhibits PLD activation and lysosomal translocation.
(A) HEK293 cells were transduced with shLRS or control lentiviruses and selected with puromycin for 3 days, followed by western blot analysis.
(B) HEK293 cells were serum- and amino acid-starved, and then stimulated with amino acids in the absence or presence of 5 μM SAR405, followed by *in vivo* PLD assays.
(C) HEK293 cells were transfected with HA-PLD1, treated as in (B), and then immunostained with anti-HA and anti-LAMP1 antibodies. Quantification of colocalization was as described in Figure 2D and 2E legends.
(D) HEK293 cells were treated as in (B) and immunostained with anti-mTOR and anti-LAMP1 antibodies. Quantification of colocalization was as described in Figure 2D and 2E legends.

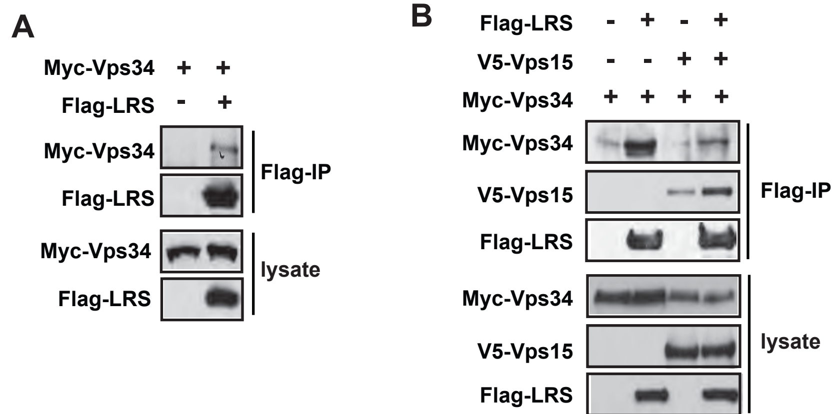

Figure S2 – related to Figure 4. Vps34-LRS interaction is independent of Vps15.
(A) HEK293 cells were transfected with Myc-Vps34 and Flag-LRS. Anti-Flag IP was performed, followed by western analysis.
(B) HEK293 cells were transfected as in (A) with or without V5-tagged Vps15, followed by anti-Flag IP and western analysis.

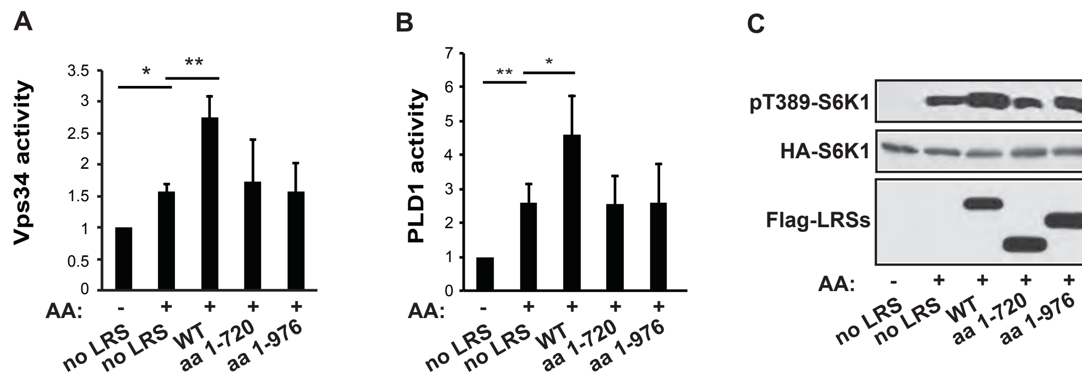

Figure S3 – related to Figure 4. Vps34-binding LRS fragments do not activate Vps34, PLD1, or mTORC1.
(A) HEK293 cells were co-transfected with bicistronic Myc-Vps34/V5-Vps15 and LRS constructs as indicated, serum starved overnight, and amino acid deprived for 2 hr, followed by amino acid stimulation for 30 min. Anti-Myc IP immunocomplexes were subjected to *in vitro* Vps34 kinase assays.
(B) HEK293 cells were co-transfected with HA-PLD1 and LRS constructs as indicated and then treated as above, followed by *in vivo* PLD assays.
(C) HEK293 cells co-transfected with HA-S6K1 and LRS constructs were treated as above and analyzed by western blotting.

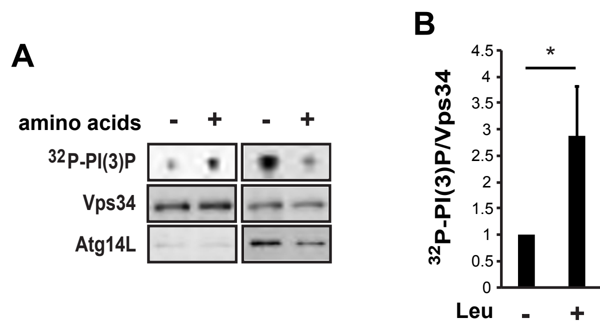

Figure S4 – related to Figure 4. Non-autophagic Vps34 is activated by amino acids.
(A) MEF cells were serum starved overnight, amino acid deprived for 2 hr, and then stimulated with amino acids for 30 min. IP was performed with anti-Atg14L (right panels) or anti-Vps34 (left panels) after preclearing the cell lysate with an anti-Atg14L antibody, followed by *in vitro* Vps34 kinase assay.
(B) MEF cells were treated as in (A) without stimulation, and subjected to IP with anti-Vps34 after preclearing with anti-Atg14L, followed by *in vitro* Vps34 kinase assay with or without 0.8 mM leucine.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies and other reagents – Antibodies were obtained from the following sources: FLAG M2 and Atg14L (for western blot) from Sigma; Myc (9E10.2) and HA (16B12) from Covance; tubulin, LRS, LAMP1, and LAMP2 from Abcam; EPRS and IRS from Abcam and Neomics; V5 from Invitrogen; Vps34 (for IP) from Echelon Biosciences; Atg14L (for IP) from MBL International; Beclin1 and Vps15 from Bethyl Laboratories; all other antibodies from Cell Signaling Technology. The PLD1 antibody was generated by Proteintech Group, Inc. using a synthetic peptide corresponding to the C-terminal sequence of PLD1. 9,10-^{3}H-oleic acid and ^{32}P-γ-ATP were from PerkinElmer. SAR405 was from APExBIO. C8-PA was from Avanti Lipids. All other reagents were from Sigma-Aldrich.

Plasmids – All cDNAs used in this study were of human sequences except S6K1 (rat). The following plasmids have been reported: HA-S6K1 (Yoon et al., 2011), Myc-S6K1 and HA-PLD1 (Fang et al., 2003); wild type Myc-Vps34 (Byfield et al., 2005); bicistronic Vps34/Vps15 (Yan et al., 2009). pCDNA-Myc-LRS, pCMV2-Flag-LRS, and pGEX4T-1-GST-LRS constructs were previously reported (Han et al., 2012) except the following. cDNAs for LRS fragments spanning amino acids 1-360, 361-720,1-720, 1-976, 721-1176, and 1-1064 (ΔUNE-L) were PCR-amplified and subcloned into the pCDNA8Flag vector via BamHI and XhoI sites. For GST- and Flag-LRS F50A/Y52A, a BamHI-KpnI fragment and an EcoRV-KpnI fragment carrying the F50A/Y52A mutations replaced the corresponding wild-type fragments in GST-LRS and Flag-LRS, respectively.

Cell lysis, immunoprecipitation, and western analysis – Cells were rinsed with ice-cold PBS and lysed in MIPT buffer as previously described (Yoon et al., 2013) except the following. For immunoprecipitation of Vps34, Beclin1 and Atg14L, cells were lysed with Mild Lysis Buffer (MLB: 10 mM Tris, pH7.5, 2 mM EDTA, 100 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Sigma)). The supernatant after microcentrifugation at 13,000 ×g for 10 min was collected and subjected to immunoprecipitation at 4 °C with various antibodies in the lysis buffer, followed by incubation with protein G-agarose. The resulting beads were washed with lysis buffer 3-5 times and used for further analysis. Immunoprecipitation for Flag-LRS was performed with M2-beads (Sigma) with MIPT buffer. Protein samples were boiled in SDS sample buffer and subjected to western analysis using horseradish peroxidase-conjugated secondary antibodies detected with Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer, Inc.). Quantification of western band intensities was performed by densitometry of x-ray film images using the software Image J.

***In vitro* Vps34 lipid kinase assay** – Vps34 immune complexes were washed sequentially with PBS containing 1% NP-40 (3 times), 100 mM Tris pH7.4 with 500 mM LiCl (3 times), and 2 times with TNE buffer (10 mM Tris, pH7.4, 100 mM NaCl and 1 mM EDTA), followed by incubation in TNE buffer containing 2 mg/ml PI, 10 μCi ^{32}P-γ-ATP, and 3.75 μg GST proteins in the presence or absence of leucine at 30 °C for 20 min. Reactions were terminated by adding 20 μl of 8N HCl, and the lipid was extracted by 160 μl of $CHCl_3$:MeOH (1:1). ^{32}P-PI(3)P was separated on a thin-layer-chromatography plate (EMD chemicals Inc.) with $CHCl_3$:MeOH:NH_4OH:Water (60:47:2:11.3) as the solvent and detected by autoradiograph. Activity was normalized to the amount of Vps34 in the immunoprecipitates determined by quantification of western blotting.

***In vivo* PLD assay –** HEK 293 cells in 12-well plates were labeled with 5 μCi ^{3}H-oleic acid per well (500 μL total volume) for one day, and subjected to various treatments as described in figure legends. Following 0.3% 1-butanol treatment for 30 min, the cells were lysed and lipids were extracted and analyzed by thin layer chromatography as described in Sun et al., 2008. To calculate recombinant PLD1 activity, the PLD activity in cells transfected with empty vector was subtracted from the activity in PLD1 transfected cells under the same conditions.

Immunofluorescence imaging – HEK293 cells cultured on poly-L-lysine-coated glass coverslips were transfected and treated as indicated in figure Legends, followed by fixation in 3.7% paraformaldehyde and permeabilization with 0.1% Triton X-100. Incubation with various primary antibodies was performed in 3% BSA/PBS at 4 ^{0}C overnight, followed by incubation with Alexa-anti-mouse 594 and Alexa-anti-rabbit 488 antibodies in 3% BSA/PBS for 30 min at room temperature. A personal deconvolution microscope system (DeltaVision; Applied Precision) was used with a 60× NA 1.4 lens to analyze the fluorescence images. Deconvolution used an enhanced ratio iterative-constrained algorithm (Agard et al., 1989). XY and Z optical displacement between different filter sets was determined experimentally using Tetraspeck fluorescent microsphere standards (Invitrogen). For the quantitative analysis of colocalization, fluorescent images were obtained using a Laser Scanning confocal Microscope 700 (LSM 700, Carl Zeiss) equipped with an LSM T-PMT camera (Carl Zeiss, LSM 700). Overlap coefficient was measured

using the ZEN2009 software. Colocalization in each sample was determined by the mean of the overlap coefficient for more than 10 cells.

SUPPLEMENTAL REFERENCES

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Yoon, M.S., Zhang, C., Sun, Y., Schoenherr, C.J., and Chen, J. (2013). Mechanistic target of rapamycin controls homeostasis of adipogenesis. J Lipid Res *54*, 2166-2173.