SUPPLEMENTAL FIGURES

Figure S1. Electrophysiological Characterization of lysoNa$_{ATP}$, Related to Figure 2. Representative recordings are in *left* and averaged sizes (at -100 mV) are in *right* in each panel. All the recordings had 1 μM PI(3, 5)P_2 in the bath unless otherwise stated. Recordings were obtained from HEK293T cells transfected GFP-tagged constructs except panel C (TPC2 without tag used) and panel F (from macrophage). (A, B) TRPMLs do not form ATP-sensitive channels. Currents were recorded from TRPML3- (*A*) or TPRML1- (*B*) - transfected endolysosomes in the presence of 1 μM (*A*) or 0.1 μM PI(3,5)P_2 (*B*). (C) LysoNa$_{ATP}$ recorded from HEK293T cells transfected with a full-length TPC2 without any tag. (D) Mg^{2+} does not inhibit TPC current. Currents were recorded with standard bath (containing 2 mM Mg^{2+}, free [Mg^{2+}] 1.94 mM) or bath with additional 1 mM Mg^{2+} (total of 3 mM, free [Mg^{2+}] = 2.91 mM). (E) Basal TPC2 current is also inhibited by ATP in the absence of added PI(3,5)P_2. Only currents > 50 pA (at -100 mV without ATP) were included for the average analysis (349 ± 137 pA at -100 mV, n = 5). (F, G) NAADP does not activate TPC channels, as shown by the lack of effect of NAADP on the inhibition of lysoNa$_{ATP}$ from mouse peritoneal macrophages (*F*) or TPC2- transfected HEK293T cells (*G*). (H) ATPγS does not inhibit lysoNa$_{ATP}$. Currents were recorded from TPC2-transfected HEK293T cells. (I) The inhibition of lysoNa$_{ATP}$ by ATP is slow. *Left*, current (at -100 mV) was continuously recorded from a TPC2-transfected HEK293T cell during solution changes. Time 0 indicates break-in. *Right*, current amplitudes normalized to that before application of ATP indicating the time courses of ATP inhibition (n = 3 or 4).

Figure S2. mTOR, but Not AMPKs, Is Required for the ATP Sensitivity of lysoNa$_{ATP}$, Related to Figure 3. Currents were recorded from macrophage endolysosomes in the presence of the AMPK inhibitor Dorsomorphin (*A*), mTOR inhibitor Torin 1 (*C*), or from TPC2-transfected AMPK1/2 double knockout embryonic fibroblasts (*B*).

Figure S3. Specificity of the Association between TPCs and mTOR, Related to Figure 4. (A-D) TPC2 does not associate with RagB, V-ATPase or lamptors. Cells were tranfected with combination of plasmids as indicated in the table above each panel. Total proteins were immunoprecipitated (IP) with antibodies indicated below each lane and blotted with antibodies indicated on the left of each panel. (A-D) show that TPC2 does not associate with RagB (*A*, lamtor 1 used as a positive control), V-ATPase (*B*, endogenous V-ATPase detected with anti-V1 Subunit D; RagB as a positive control), lamptor 1 (*C*, RagB as positive control), or lamptor 2 (*D*, RagB as positive control). (E) TRPML1-3 do not associate with mTOR. Immunprecipitates with anti-GFP from cells transfected with plasmids as indicated were blotted with anti-mTOR (*upper*) or anti-GFP (*lower*).

Figure S4. LysoNa$_{ATP}$s Are Not Required for mTOR Activities, Related to Figure 6. (A) Total cell lysates from cultured hepatocytes treated with or without insulin (100 nM, 1 hr), amino acid (1x, 1 hr) and rapamycin (50 nM, 1 hr) as indicated were blotted with anti-pP70S6K (*upper*) or β-actin (*lower*). (B) Immunostaining using anti-mTOR antibody with WT (*upper*) and TPC1/2 dKO (*lower*) MEF cells. Cells in the left panels were starved in amino acid-depleted medium for 50 min. Cells in the right panels were re-fed with amino acids for 10 min after starvation. Scale bars, 5 μm.

Figure S5. Phenotype Analysis of TPC1/2 Double Knockout Mutant Mice, Related to Figure 7. (A-C) Autophagy analysis. (A) Total liver proteins from fed and fasted WT and TPC1/2 dKO mice were blotted with anti-LC3 (*upper* panel) and anti-GAPDH (*lower* panel, loading control). (B) Similar to (*A*), but from isolated hearts with or without oxygen-glucose deprivation (OGD) perfusion. Two pairs of WT and dKO are shown. OGD perfusion was done with Langendorff perfusion for 45 min (Matsui et al. 2007). (C) The mCherry- and GFP-tagged LC3 (tandem tag, illustrated; Kimura et al., 2007; Pankiv et al., 2007) was transfected into WT (*upper* panels) and TPC1/2 dKO (*lower* panels) macrophages. Cells were starved for 1 hour and imaged for GFP (*left* panels) and RFP (*middle*). *Right* panels are the merged signals between GFP and RFP. GFP- and RFP- positive puncta indicate autophagosomes before fusing with lysosomes. RFP- positive but GFP-negative puncta (see right panels) indicate fusion between autophagosomes and lysosomes. Scale bars, 5 μm. (D) Lysosomal amino acid efflux assay with ^{14}C-labeled lysine and liver lysosomes. *, $p<0.05$; **, $p<0.01$. (E) Rotarod tests performed before (days 1-3) and during (days 7 and 8) fasting. The time at which each animal fell off the rod was recorded and averaged. Ten WT and 11 dKO mice were tested.

EXTENDED EXPERIMENTAL PROCEDURES

cDNA Constructs Used in Transfection

Unless otherwise stated, all the channel clones were GFP-tagged for the identification of channel protein-expressing endolysosomes used for patch clamp recordings. Human isoforms were used in all the experiments except Figure 5H where the mouse TPC2 (mTPC2) was also used. TPC1, TPC2, TRPML1, TRPML2 and TRPML3 were cloned into the HindIII/EcoRI (TPC2), HindIII/BamHI (TPC1), or KpnI/SmaI (mTPC2, TRPML1, TRPML2, TRPML3) sites of peGFP-C1 (TRPML1, TRPML2, TRPML3), peGFP-N1 (TPC1), or peGFP-C3 (TPC2, mTPC2) vector. Lamtors1-3 were amplified from human liver cDNA with primers containing HA or Flag tag sequences and were subcloned into the EcoRI/XbaI sites of pcDNA3. All the clones were confirmed with sequencing. The human wt mTOR, mTOR-S2035T, mTOR-S2035T/D2357E, wt RagB, $RagB^{GDP}$ (T54L) and $RagB^{GTP}$ (Q99L) clones were from Addgene (plasmid # 26603, 26604, 26605, 19313, 19314, 19315, respectively) (Sancak et al., 2010; Sancak et al., 2008; Vilella-Bach et al., 1999). Transfection was done with Lipofectamine LTX or Lipofectamine 2000 (Invitrogen).

Cell Culture

HEK293T cells were cultured in DMEM (Gibco) medium supplemented with 10% FBS (Lonza) and 1x Glutamax (Invitrogen) at 37°C in a humidified CO_2 incubator. Cardiac myocytes and fibroblasts were isolated from P0 pups. Hearts were quickly dissected out and cut into small pieces in ice-cold Ca^{2+} free D-Hank's medium. The pieces were digested by shaking at 250 rpm for 5 min at 37°C in D-Hank's medium containing 0.08% (W/V) collagenase (type II, Worthington) and 1% BSA (Sigma-Aldrich). The supernatant was mixed with 10% FBS (V/V) to stop the digestion, and centrifuged at 800 rpm (68 x*g*) for 5 min at 4°C. Cells were plated onto coverslips and cultured in DMEM/F-12 (Gibco) supplemented with 10% FBS (Lonza) and 1x Pen/strep (Invitrogen) at 37°C in a humidified CO_2 incubator. Beating myocytes used for recordings were visually identified. Fibroblasts were cultured for 4 passages before experiments. Peritoneal macrophages were extracted from sacrificed adult mice with 10 ml ice-cold PBS injected into the peritoneal cavity. Pelleted cells were plated onto coverslips and cultured in DMEM supplemented with 20% FBS and 1x Pen/strep at 37°C in a humidified CO_2 incubator.

Liver hepatocytes used for electrophysiological recording were cultured from P0 pups. Liver was quickly dissected out and cut into small pieces in ice-cold HBSS (Gibco) containing 0.2 mM EDTA. Tissue was shaken at 80 rpm at 37°C for 10 min, transferred into digestion solution containing 1 mM $CaCl_2$ and 100 unit/ml collagenase (type 4, Worthington) in HBSS, and shaken for another 10 min. Digestion was stopped by adding 10% FBS. Cells were dissociated by pipette, plated in DMEM/F12 supplemented with 10% FBS and 1x Pen/strep, and cultured at 37°C in a humidified CO_2 incubator for more than 24 hours before recording. Hepatocytes used for biochemical studies were isolated from adults following previously described protocols (Glick et al., 2012) and cultured in serum-free William's E medium supplemented with 1x Pen/strep.

Protein Chemistry

For the experiments testing interaction between TPCs and mTOR (Figures 4, 5 and S3E), transfected HEK293T cells were lysed in ice-cold lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1% NP-40, and one tablet of complete protease inhibitors (Roche) per 25 ml. Lysates were cleared by spinning at 13,200 rpm (18,506 x*g)* for 10 minutes. For Western blot, cleared lysates were resolved with 4–12% NuPAGE Bis-Tris gels (Invitrogen), and analyzed with anti-mTOR (1:1000, Cell signaling, #2983) or anti-GFP (1 μg/ml, Santa Cruz, sc-9996) antibody. For immunoprecipitation, cell lysates were incubated with protein A agarose (Invitrogen) at 4 °C for 10 min. The cell lysates were divided into two groups, one used as total cell lysate input, and the other for immunoprecipitation with anti-GFP (2 μg/ml, Invitrogen, #A11120) at 4°C for 2 h, followed by an additional 2 h incubation with protein A agarose. Precipitates were washed 3 times with cold lysis buffer and eluted with 1xLDS sample buffer (Invitrogen, NP0007) supplemented with 100 mM DTT. Samples were heated at 70 °C for 10 min, resolved in 4–12% NuPAGE Bis-Tris gels, and analyzed with immunoblotting using anti-mTOR (1:1000), -GFP (1 μg/ml, Santa Cruz, sc-9996) or -GAPDH (2 μg/ml, Millipore, MAB374) antibody. HRP-conjugated secondary antibody from the Clean-Blot kit (Pierce) was used in ECL detection to minimize contaminating signals from the primary antibodies used in immunoprecipitation.

For the experiments testing interaction between TPC2 and V-ATPase, transfected cells were lysed in ice-cold buffer containing 150 mM NaCl, 40 mM HEPES (pH 7.4), 2 mM EGTA, 2.5 mM $MgCl_2$, 1% Triton X-100, and one tablet of mini EDTA-free protease inhibitors (Roche) per 10 ml (Zoncu et al., 2011). Cell lysates were immunoprecipitated with anti-GFP (4 μg/ml, Invitrogen #A11120) or anti-Flag (4 μg/ml, Sigma F3165) at 4°C for 2 h. Immunoblots were analyzed using anti-V-ATPase D1 (1 μg/ml Santa Cruz, sc-166218). HRP-conjugated sheep anti mouse secondary antibody (GE Healthcare, NA931V) was used in ECL detection. To probe the interaction between TPC2 and RagB or lamtor 1/2/3, transfected HEK293T cells were lysed in ice-cold buffer containing 40 mM HEPES (pH 7.4), 10 mM pyrophosphate, 10 mM glycerophosphate, 1% Triton X-100 and one tablet of complete protease inhibitors (Roche) per 25 ml, as described before (Sancak et al., 2010). Cell lysates were immunoprecipitated at 4°C for 2 h with anti-GFP (4 μg/ml, Invitrogen #A11120), anti-Flag (4 μg/ml, Sigma F3165) or anti-HA antibody (4 μg/ml, Santa Cruz sc-805). Immunoblots were analyzed using anti-Flag (5 μg/ml), -GFP (1 μg/ml, Santa Cruz, sc-9996) or -HA (1 μg/ml) antibody. HRP-conjugated secondary antibody from the Clean-Blot kit (Pierce) was used in ECL detection to minimize contaminating signals from the primary antibodies used in immunoprecipitations.

Immunohistochemistry

Mouse embryonic fibroblast (MEF) cells were cultured from WT and dKO embryos as described before (Xu, 2005), and were plated on glass coverslips (12 mm diameter) one day before the assay. Cells were incubated in starvation buffer (in mM, 110 NaCl, 45 $NaHCO_3$, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, pH7.4) for 50 min, and were divided into two groups. One was directly used for immunostaining and the other was stimulated with 10X amino acid in culture medium for 10 min before immunostaining. Cells were washed once with PBS, fixed in 4% PFA at room temperature for 15 min, and washed twice with PBS. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min, and blocked for 1 hour with 5% dry milk in PBST containing 0.1% Tween-20 in PBS. The coverslips were incubated with rabbit mTOR antibody (1:200, Cell Signaling) in 3% dry milk/PBST at 4°C overnight. After 3 times wash (10 min each) with PBST, the coverslips were incubated with TRITC-conjugated anti rabbit secondary antibody (1:25, Pierces, #31670) for 1 hour at room temperature in the dark and washed again with PBST for 3 times. Washed coverslips were mounted on slides with Fluoromount (Sigma, F4680) and imaged

with an Olympus IX71 microscope equipped with a VT-Hawk confocal unit (VisiTech International Ltd, United Kingdom) using a 60X water immersion objective.

Lysosomal Amino Acid Efflux Measurement

Lysosome preparation: Lysosomes were prepared from mouse liver using differential centrifugation following previously described protocols (Reeves, 1979). Briefly, liver from each adult mouse (12-13 week old male) was homogenized with a Dounce homogenizer in 8 ml homogenization buffer (HB) containing 250 mM sucrose, 1 mM Na_2EDTA, 10 mM HEPES (pH 7.0). The homogenate was centrifuged at 1,000x*g* for 10 min at 4°C. Supernatant was kept on ice; the pellet was homogenized again in 7 ml HB and spun again to obtain the supernatant. The two supernatants were combined and centrifuged at 20,000x*g* for 20 min at 4°C. The pellet was resuspended in buffer containing 250 mM KCl and 1 mM MOPS (pH7.4). After spinning at 1,600x*g* for 10 min, the supernatant was diluted by 2.5x volume with buffer containing 450 mM sucrose, 0.5 mM Na_2EDTA and 5 mM HEPES (pH7.2), and centrifuged again at 25,000x*g* for 10 min. The pellet containing crude lysosomes was resuspended in 200 μl uptake buffer (250 mM sucrose, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.0) and centrifuged at 600x*g* for 2 min. The supernatant was kept on ice; the pellet was resuspended in (100 μl) uptake buffer by passing through a 30G needle for 30 times, and then combined with the supernatant. An aliquot of the crude lysosome was used for total protein determination with protein assay and the rest was kept on ice and used for amino acid efflux assays within 12 hours.

Preparation of amino acid methyl esters: ^{14}C-labeled lysine- and arginine- methyl esters were prepared from ^{14}C-labeled lysine and arginine following previously described methods (Reeves, 1979; Steinherz et al., 1982). Briefly, 12.5 μCi ^{14}C-labeled amino acid (from Perkin Elmer) was transferred into a 25-ml glass conical flask, dried under stream of nitrogen, dissolved in 3 N methanolic HCl (Sigma) and incubated for 24 hours at room temperature. The resulting amino acid-methyl ester was dried under stream of nitrogen, washed with methanol and dried again before being dissolved in 500 μl methanol, followed by volume reduction to 250 μl through evaporation. Shortly before efflux assay, appropriate amount was transferred into a 1.5 ml tube, dried under stream of nitrogen, and dissolved in uptake buffer (final concentration 0.005 μCi/μl).

Amino acid efflux assay: The crude lysosomes were dispensed in 50 μl aliquots, each vial containing 100 μg of protein-equivalent amount of lysosomes. To initiate amino acid ester loading, 50 μl of amino acid methyl ester (0.25 μCi) in uptake buffer was added to each lysosome-containing vial and incubated at 37°C for 30 min (experimentally determined to be optimal for loading). At the end of loading, each vial was diluted into 5 ml pre-warmed efflux buffer (50 x dilution) containing 250 mM sucrose, 5 mM $MgCl_2$, 20 mM HEPES (pH 7.0), 0.1 μM $PI(3,5)P_2$, and, in some assays, ATP (0.1 or 2 mM as indicated in the figures), mixed well and incubated at 37°C. At each given time point, a 5 ml efflux assay reaction was combined into 5 ml ice-cold PBS, filtered immediately through a Whatman filter (GF/F) under vacuum, and washed twice with 5 ml ice-cold PBS. For background count, lysosomes and amino acid methyl ester incubated separately were mixed and filtered immediately (without loading). The filter was soaked in scintillation cocktail overnight and counted using a Beckman LS6500 scintillation counter. Background-subtracted isotope counts were normalized to that obtained at efflux time point 0.

Behavior Tests

Male mice (9-11 weeks) were used for the tests. For fasting, animals had access to water but not food. Rotarod tests were performed using a 4-lane rotarod (Rotamex-5, Columbus Instrument). One day before the start of test sessions, each mouse was placed onto the rod for acclimation until it could remain on the rotarod for 30 s at 4 rpm. For test sessions, the rod rotation speed started at 4 rpm and increased 1 rpm every 8 s until the animal fell off. Test data from 3 daily sessions (1 h apart) were averaged for each animal. Treadmill tests were performed using a 6-lane treadmill (Exer-3/6, Columbus Instrument) set at a 10 degree incline. Before the test sessions, each mouse was allowed to acclimate to the treadmill by running for 5 min at 10 m/min each day for 3 days. For test sessions, the belt speed was first increased to 10 m/min over 1 min. The animals were then stimulated (electrical shock of 0.47 mA at 1 Hz delivered in the resting grid in the rear end of the treadmill) to run at 10 m/min for 5 min. The speed was then increased by 1 m/min over each min. Electrical stimuli were turned off immediately after the animal was exhausted, defined by a lack of an active attempt to reengage the moving belt after 10 s of stimulation.

SUPPLEMENTAL REFERENCES

Kimura, S., Noda, T., and Yoshimori, T. (2007). Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 3, 452-460.

Matsui, Y., Takagi, H., Qu, X., Abdelatif, A., Sakoda, H., Asano, T., Levine, B., and Sadoshima, J. (2007). Distinct roles of autophagy in heart during ischemia and reperfusion: roles of AMP-activated protein kinase and beclin 1 in mediating autophagy. Circ Res 100, 914-922.

Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J.-A., Outzen, H., Øvervatn, G., Bjørkøy, G., and Johansen, T. (2007). p62/SQSTMI binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282, 24131-24145.

Reeves JP (1979) Accumulation of amino acids by lysosomes incubated with amino acid methyl esters. J Biol Chem 254: 8914-8921.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A. L., Nada, S., and Sabatini, D. M. (2010). Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell *141*, 290-303.

Sancak, Y., Peterson, T. R., Shaul, Y. D., Lindquist, R. A., Thoreen, C. C., Bar-Peled, L., and Sabatini, D. M. (2008). The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science *320*, 1496-1501.

Steinherz R, Tietze F, Raiford D, Gahl WA, and Schulman JD (1982) Patterns of amino acid efflux from isolated normal and cystinotic human leucocyte lysosomes. J Biol Chem 257: 6041-6049.

Vilella-Bach, M., Nuzzi, P., Fang, Y., and Chen, J. (1999). The FKBP12-Rapamycin-binding Domain Is Required for FKBP12-Rapamycin-associated Protein Kinase Activity and G_1 Progression. J Biol Chem *274*, 4266-4277.

Xu, J. (2005). Preparation, culture, and immortalization of mouse embryonic fibroblasts. Curr Protoc Mol Biol *Chapter 28*, Unit 28.21.

Zoncu, R., Bar-Peled, L., Efeyan, A., Wang, S., Sancak, Y., and Sabatini, D. M. (2011). mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. Science *334*, 678-683