Supporting Information

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SI Material and Methods

Plasmids and Antibodies. The following vectors were used in this study. All Barkor mutants used for the autophagosome binding assays were cloned into the Bgl II and EcoR I sites on hrGFP-N1, and a 3xFlag tag was inserted between the Sal I and BamH I sites. hrGFP–Barkor/Atg14(L) autophagosome targeting sequence (BATS) triple R mutation (W484R, F485R, Y488R) was cloned from full-length Barkor using the following primers: P1: GCGAATTCGgctggagaatcagatgagagcg and P2: GCGAATTCGAacggtgtccagtgcgagct ttacgccgggaggtcaccga. Beclin 1 was inserted into hrGFP-N1 between Sac I and Hind III, and hrGFP-Beclin 1-BATS was generated by inserting BATS at the EcoR I site of hrGFP-Beclin 1. pcDNA3-Myc-LC3 was described previously (1). pCDNA3.1-Myc-Barkor and pcDNA3.1-Barkor Δ10aa were cloned in between the EcoR I and Kpn I. pcDNA3.1-Myc-Beclin 1 was made by insertion of Beclin 1 into the NotI and BamHI of pcDNA3.1-Myc. pDsred-ER was purchased from Clontech. Antibodies used in this study include: FLAG M2, c-Myc (A-14, 9E10), β tubulin, GFP, and LC3, which were described previously (1).

Stably Expressed Cell Lines. Cell lines that stably express Myc-LC3 and Myc-Beclin 1 were described previously (1). pCDNA3.1-Myc-Barkor and pCDNA3.1-Myc-Barkor Δ10aa were transfected into U_2OS cells, and 24 h after transfection, cells were exposed to 1 mg/mL G418 for 10 h. Next, normal DMEM medium was added to the cells. Two weeks later, colonies were picked, and the expression of Barkor wild-type or mutant was tested by anti-Myc antibody through Western blotting.

Immunohistochemistry. Cells were grown on cover slips (Fisher) and were subjected to different treatments followed by fixation in 4% paraformaldehyde for 20 min. Next, the cells were permeabilized with 0.4% Triton X-100 for 20 min at room temperature. Anti-c-Myc (Santa Cruz, A-14) at a concentration of 0.5 μg/mL and Rhodamine Red goat antiRabbit (Jackson) was diluted in 10% goat serum [100% goat serum diluted 10 times by Dulbecco's PBS (DPBS)] with 0.1% Tween 20 and was used as primary and secondary antibodies, respectively. Cells were washed by DPBS 10 min each for five times at room temperature after probing with each antibody. After the final wash, cover slips were mounted onto glass slides (CORNING). Images of immunostaining were acquired using a microscope (Axio Observer Z1; Carl Zeiss, Inc.), camera (AxioCamMR3 or MrC5; Carl Zeiss, Inc.), and Axiovision software (Carl Zeiss, Inc.). Acquisitions were performed at room temperature using 100× 1.3 NA EC Plan-Neofluar objectives (Carl Zeiss, Inc.). Confocal pictures were acquired at room temperature using a multiphoton confocal microscope (LSM510 NLO; Carl Zeiss, Inc.) fitted on an inverted microscope (Axiovert M200; Carl Zeiss, Inc.) equipped with C-Apochromat 100× NA 1.3 oil immersion objectives (Carl Zeiss, Inc.). The datasets generated were merged and displayed with the LSM510 software (Carl Zeiss, Inc.).

Transmission Electron Microscopy. Cells were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h followed by treatment with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h. Samples were blocked with 0.5% aqueous uranyl acetate overnight and treated by low-temperature dehydration and infiltration with a graded series of Epon/Araldite and were embedded in 100% Epon/Araldite. Thin sections (70 nm) were cut with a diamond knife and stained with Reynolds lead citrate and analyzed by an FEI Tecnai 12 transmission electron microscope.

Autophagy Assay. For immunostaining, U_2OS cells were either subjected to 200 μg/mL chloroquine for 2 h or to 2 μg/mL Rapamycin for 4 h. Starvation was done by incubating cells in Earle's balanced salt solution (EBSS) for 30 min. 50 μM Bafilomycin was used for 2 h to block lysosome degradation.

Live Cell Image. U_2OS cells transfected with different DNA constructs were plated onto glass coverslips (fisher) and fastened in a secured chamber. Live cell images were taken using a microscope (IX-81; Olympus) equipped with a temperature-controlled enclosure (Precision Control Weather Station), a 100× NA 1.4 objective, and a camera (Orca-ER). Movie S1 was taken with 30 min EBSS treatment, each image was taken with a 10-s interval. Two-color images were obtained by sequential switching between RFP and GFP filters. To make the two colors as synchronized as possible, a 2-s interval was used when taking two-color live images; all cells were treated with EBSS. All image processing was performed using MetaMorph software (MDS Analytical Technologies).

Protein Purification. BATS or BATS WFY mutant was cloned into PGEX4T-1 between ECORI and NOTI. Expression vectors were first transformed into Rosseta competent *Escherichia coli* cells; single colony was inoculate into 100 mL LB medium with 1 mg/mL ampicilin at 37 °C overnight. Bacteria was then transferred into 1 L LB and cultured at room temperature until OD600 reached 0.6. GST expression was induced by 500 μM isopropyl beta-D-1-thiogalactopyranosid (IPTG) for 2 h at room temperature. Bacteria were collected by centrifugation at 4,000 rpm (Thermo Scientific, Sorvall SA-600) for 30 min, resuspended in 30 mL lysis buffer (500 mM NaCl 10 mM Tris-Cl pH 7.5, 1% Triton X-100, protease inhibitor cocktail), then lysed by sonification. Cell lysate was collected by centrifugation at 10,000 rpm (Thermo Scientific, Sorvall SA-600) for 30 min. Packed glutathione Sepharose (GE) (0.8 mL) was used to conjugate the GST protein. Glutathione (10 mM) was used to do the final elution. Full-length Barkor was purified from sf9 cell according to Invitrogen Bac-to-Bac Baculovirus expression system. Barkor was first cloned into pFASTBAC donor plasmid that had been first tagged with ZZ (the tandem repeat dimer of the modified immunoglobulin binding domain of protein A of *Staphylococcus aureus*) followed with tobacco etch virus (TEV) cleavage site and 6xhis. pFASTBAC with full-length Barkor was then transformed into DH10α competent cells to get Bacmid DNA. White colonies from plate (50 μg/mL kanamycin, 7 μg/mL gentamicin,10 μg/mL tetracycline, 100 μg/mL Bluo-gal, 40 μg/mL IPTG) was selected to yield Bacmid DNA. The Bacmid DNA with the right gene insertion was used to infect sf9 cell to reach an eventual 1 L with about 10^8 cells. Cells were harvested by centrifugation, resuspended in hypotonic buffer (20 mM Tris-HCl pH7.5, 0.1% Triton X-100, 5 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, protease inhibitors) on ice for 15 min, followed by douncing with pestle (Wheaton) 30 times, high salt buffer (20 mM Tris-HCl pH7.5,0.1% Triton X-100,250 mM NaCl,0.5 mM DTT 20 mM Glycerol) was added to reserve the solution to physiological salt level, and cell lysate was incubated with IgG beads to allow the conjugation of ZZ tag to the beads. TEV cleavage was applied to do the final elution. All purified proteins were dialyzed in HKM

buffer (20 mM Hepes, pH 7.0, and 160 mM KOAc, 5 mM $MgCl_2$) before being used in any assay.

Liposome Preparation. Liposomes composed of brain lipid extract (type 1, folch fraction 1; Sigma-Aldrich) was first dissolved in chloroform to reach a final concentration of 10 mg/mL. Ten microliters of chloroform containing lipid was then transferred to a glass tube and dried by a rotavapor.One milliliter of HK buffer (20 mM Hepes, pH 7.0, and 160 mM KOAc) either with or without 0.3 M sucrose was applied to dried lipids, and the suspension was incubated at 37 °C for 2 h followed by 21 passages through different sizes (100, 200, 400, and 800 nm) of Nuclepore polycarbonate membrane (Whatman). Final liposome concentration was 0.1 mg/mL. DOPC, DOPS, DOPE, PI3P, PI4P, PI5P, and PI(4,5)P2 were purchased from Avanti; lipids with different combination were mixed in chloroform and processed as described above. DOPC and DOPE was used as a 1:1 ratio combining different amount of PI3P, PI(4,5)P2. Final liposome concentration was 350 μM. Taxes Red-DHPE (Invitrogen) was used as an indicator.

Liposome Binding. Five micrograms of protein in 30 μL HKM buffer (20 mM Hepes, pH 7.0, and 160 mM KOAc, 5 mM $MgCl_2$) was incubated with 100 μL sucrose-loaded liposomes. Reaction was carried out by incubation on ice for 20 min followed by 10 min incubation at 37 °C water bath. Liposomes were then sedimented at 100,000 × *g* in a Beckman Coulter TLA 100 rotor at 4 °C for 25 min. Supernatant was removed thoroughly, and sedimented liposomes were solubilized in 1xSDS. Two percent of each fraction was subjected to SDS-PAGE and analysis by Western blotting.

1. Sun Q, et al. (2008) Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. *Proc Natl Acad Sci USA* 105:19211–19216.

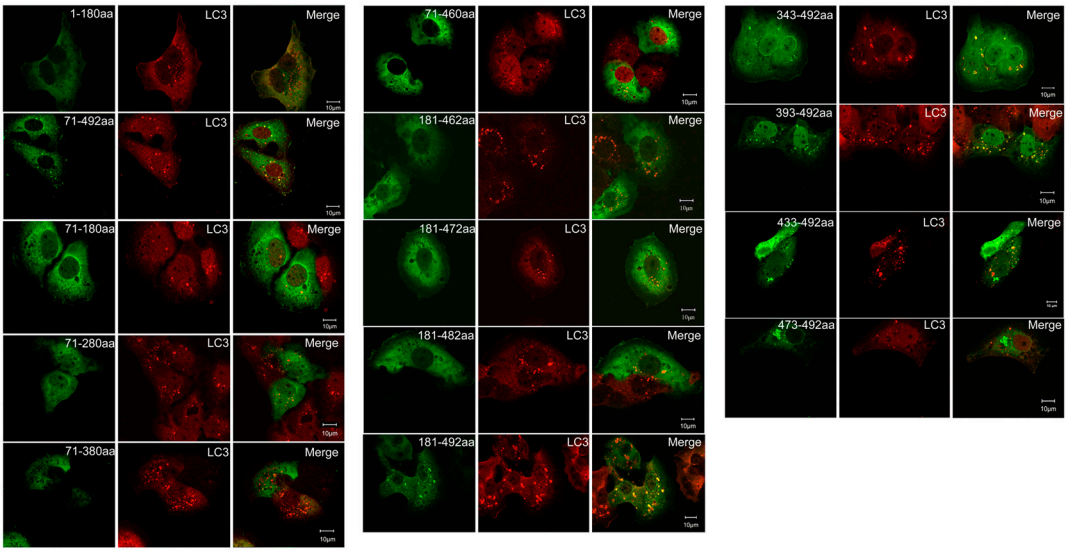


Fig. S1. Mapping results of Barkor/Atg14(L) autophagosome targeting sequence. Different Barkor mutants tagged by hrGFP were transfected into U_2OS cells expressing Myc-LC3, Forthy-eight hours after transfection, cells were treated with 200 μM chloroquine for 2 h. Myc-LC3 was stained by Rhodamine Red fluorescent antibody. Images were taken by laser confocal microscope.

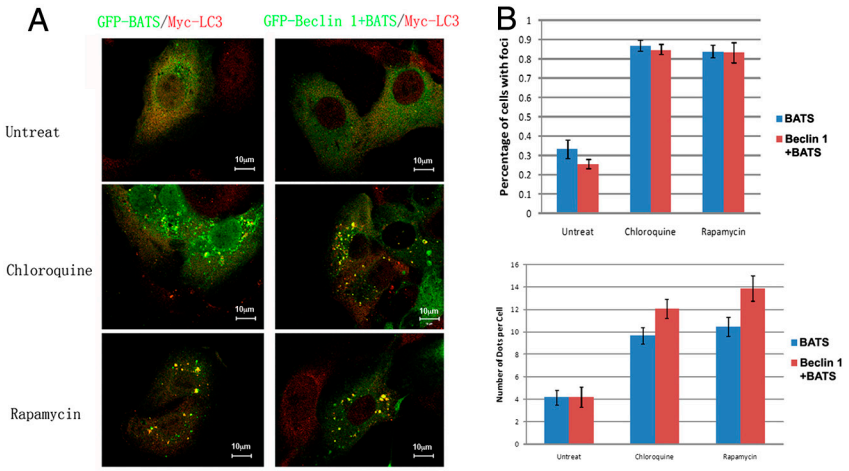


Fig. S2. BATS and Beclin1-BATS puncta are induced by autophagic stresses. U_2OS cells expressing Myc-LC3 were transfected with either GFP-BATS or GFP-Beclin1-BATS. Forty-eight hours after transfection, cells were left untreated or treated as follows: 200 μM chloroquine for 2 h, 2 μM rapamycin for 4 h, respectively. Myc-LC3 was stained by Rhodamine Red fluorescent antibody. (*B*) The number of GFP puncta in *A* was calculated, more than 200 cells were counted each time, and the experiment was repeated three times independently. Beclin1-BATS: beclin1 and BATS were constructed into the same plasmid to achieve a chimeric protein.

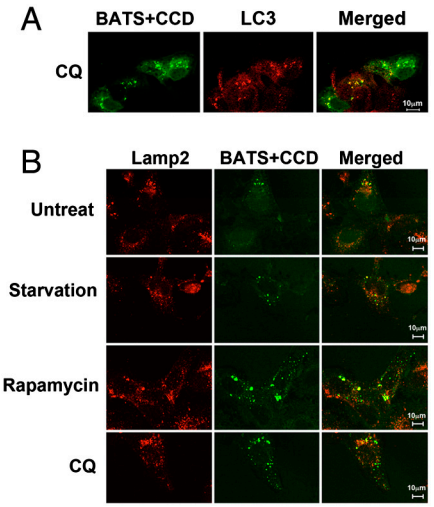
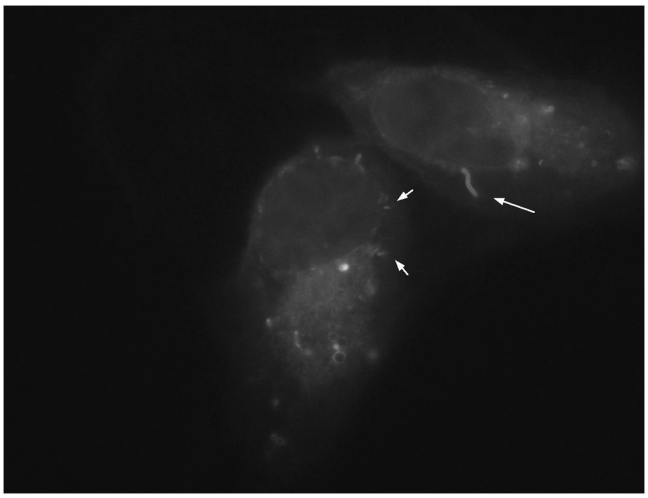
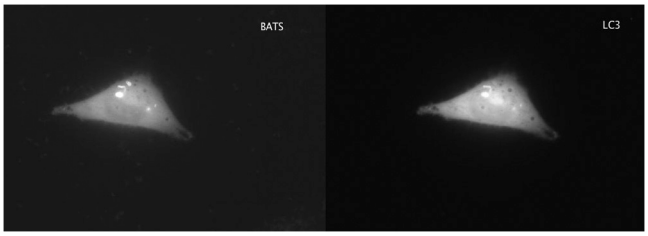


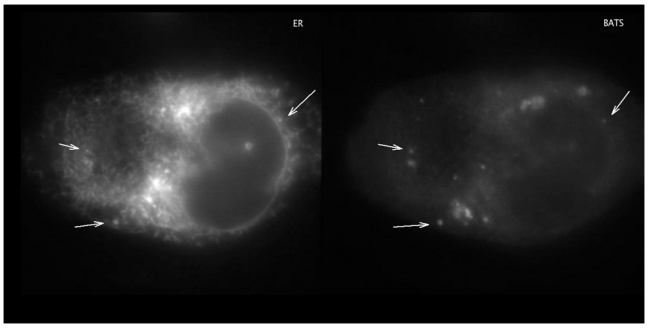
Fig. S3. BATS-CCD chimeric protein colocalize with LC3 and lamp2 under chloroquine treatment. (*A*) U_2OS cells expressing Myc-LC3 were transfected with GFP-BATS-CCD chimeric DNA construct. Forty-eight hours after transfection, cells were treated with 200 μM chloroquine for 2 h. LC3 was stained with Rhodamine Red fluorescent antibody. (*B*) U_2OS cells were transfected with GFP-BATS-CCD chimeric DNA construct. Forty-eight hours after transfection, cells were treated as follows: untreated, EBSS 0.5 h, 2 μM rapamycin 4 h, 200 μM chloroquine 2 h. Lamp2 and Rhodamine Red fluorescent antibodies were used to label lysosomes. Images were captured by laser confocal microscope.



Movie S1. Single channel image of hrGFP-BATS during starvation showing formation of tubule structures. U_2OS cells were transiently transfected with hrGFP-BATS and imaged at 1 frame per 10 s for 30 min in EBSS medium. The arrows indicate the narrow tubules decorated by BATS upon EBSS treatment.
Movie S1 (AVI)



Movie S2. Double channel image of hrGFP-BATS and Tomato-LC3 during starvation showing LC3 locates at BATS positive narrow tubules. U_2OS cells were transiently transfected with both DNA construct and images at 1 frame per 2 s; dual channel images were taken by sequentially switching RFP and GFP filters; cells were cultured in a chamber with 2 mL EBSS.
Movie S2 (AVI)



Movie S3. Double channel image of hrGFP-BATS and Dsred2-ER during starvation showing BATS partially colocalized with ER-derived puncta. U_2OS were transiently transfected with both DNA construct; cells were set in a chamber with 2 mL EBSS; images were taken with dual laser channels with 1 frame per 10 s. Arrows indicates three representative puncta with both BATS and ER signals.
Movie S3 (AVI)