Supporting Information

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Experimental Procedures

Reagents. Anti-Barkor antibody was generated by immunizing rabbits with a bacterially expressed FLAG-tagged Barkor (1–91 aa) fusion protein by Abmart and Yumay Chen from the University of Texas Health Science Center at San Antonio. The antibody was affinity-purified by the Affinity-10 beads conjugated with the immunogen. The Beclin 1 antibody was generated by Abmart or purchased from Abgent. Other antibodies used in this study include anti-FLAG (M2; Sigma), anti-HA (Roche), anti-Myc (9E10; Santa Cruz Biotechnology), anti-tubulin (Santa Cruz Biotechnology), anti-Bcl-2 (Santa Cruz Biotechnology), anti-UVRAG (Abgent), anti-EEA1 (Abcam), and anti-LC3 (Sigma). pCDNA5/FRT/TO (Invitrogen) was modified by introducing the coding sequence of ZZ tag (between HindIII and BamHI) and 3×FLAG tag (between NotI and XhoI) and designated as pZZ-FLAG. The Beclin 1 coding sequence was inserted between BamHI and NotI to generate the reading frame of ZZ-Beclin 1-FLAG, which was used for setting up a stable cell line.

Cell Culture, Cell Transfection, and Cell Lysate Preparation. 293T and U_2OS cells were cultured in DMEM supplemented with 10% FBS. For the tetracycline-inducible cells, Tet-approved FBS (Clontech) was used. Cell transfection was performed with Lipofectin 2000 (Invitrogen) or GenEscort (Wisegen) according to the protocol provided by the manufacturer. Whole-cell lysates (WCLs) used for immunoprecipitation and immunoblotting of different cell lines were prepared in tandem affinity purification (TAP) buffer [20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, protease inhibitor mixture (Roche)].

Coimmunoprecipitation Assay. Cells were lysed with TAP buffer as described above. WCLs obtained by centrifugation were incubated with 2 μg of antibody for 4 h and probed by protein A–Sepharose beads (Amersham Biosciences) for 4 h at 4 °C. The immunocomplexes were then washed with TAP buffer three times and subjected to SDS/PAGE. Immunoblotting was performed following standard procedures.

Immunofluorescence Staining. Cells were transfected with different plasmids. Twelve hours after transfection, cells were trypsinized and transferred to 6-well dishes with coverslips. Twenty-four hours later, cells grown on coverslips were fixed with 4% paraformaldehyde solution in PBS at room temperature for 20 min. After permeabilization with PBS buffer containing 0.1% Triton X-100 at room temperature for 20 min, cells were incubated with primary antibodies (anti-Myc or anti-EEA1) at 37 °C for 2 h. After washing with PBS buffer containing 0.1% Triton X-100, cells were incubated with rhodamine red-conjugated secondary antibodies at 37 °C for 2 h. Slides were examined by using a laser scanning confocal microscope (Zeiss LSM 510 META UV/Vis).

Transmission Electron Microscope Analysis. For electron microscopy, cells were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h followed by 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h. Samples were enblocked with 0.5% aqueous uranyl acetate overnight and treated by low-temperature dehydration and infiltration with a graded series of Epon/Araldite, which was followed by the embedment in 100% Epon/Araldite. Thin sections (60 nM) were cut and stained with Reynalds lead citrate and analyzed with a FEI Tecnai 12 Transmission electron microscope.

Establishment of ZZ-Beclin 1–3×FLAG Overexpression Stable Cell Line. The ZZ-Beclin 1–3×FLAG overexpression stable cell line was obtained according to the protocol provided by the manufacturer. Briefly, the pFRT/lacZeo vector was transfected into U_2OS^{TetR} cells constitutively expressing Tet repressor. Single colonies were picked and screened for LacZ activity. Cell line 28, with moderate LacZ activity, was selected for establishing the overexpression cell line. The pZZ-Beclin 1–3×FLAG vector was transfected into line 28 U_2OS cells, and the selection was performed with DMEM supplemented with 10% FBS, 0.5 μg/mL blasticidin, 50 μg/mL hygromycin, and 1% penicillin/streptomycin. Single colonies were picked and screened for the inducible expression of ZZ-Beclin 1-FLAG upon doxycycline (DOX) treatment. Clone 3 presenting the best inducibility was used for large-scale culture and subsequent purification.

Inducible shRNA Cell Line. The shRNA sequences were designed with "siRNA Target Finder" provided by Ambion and were cloned into BglII and HindIII sites of pSuperior.puro vector (Oligoengine). The inducible shRNA cell lines were established according to the protocol. The shRNA coding sequence for human Beclin 1 knockdown is GATCCCC**GGGTCTAAGACGTCCAACA**TTCAAGAGATGTTGGACGTCTTAGACCCTTTTTA, and for Barkor it is GATCCCC**GAAGGAAAGGTTAAGCCGA**TTCAAGAGATCGGCTTAACCTTTCCTTCTTTTTA. The targeted sequences of Beclin 1 and Barkor are underlined. The inducible knockdown stable cell lines were generated by using U_2OS^{TetR} cells. To measure the knockdown efficiency, cells were treated with 1 μg/mL DOX for 3 days before Western blotting was performed.

Tandem Affinity Purification of Beclin 1 Complex. The tandem affinity purification strategy to fractionate the Beclin 1 complexes from human cells was performed as follows. The stable cell line capable of expressing ZZ-Beclin 1–3×FLAG upon DOX induction was obtained. The lowest dose of DOX to induce expression of exogenous Beclin 1 close to endogenous level was chosen to apply for complex purification. Thus, the cells were grown in DMEM with 10% FBS plus 1% P/S and harvested near confluence. The cell pellet was washed with chilled PBS three times and then suspended in TAP buffer. The resuspended cell pellets were gently vortexed for 1 min after a 30-min incubation on ice. The homogenate was centrifuged for 20 min at 10,000 × *g*. The supernatant was transferred to a fresh tube. Then 0.8 mL of packed IgG beads was added to the supernatant, followed by gentle rotation overnight in 4 °C room (12–16 h). The bound protein was eluted by TEV protease cleavage and further purified by anti-FLAG antibody-conjugated beads. The final eluates from the FLAG beads with FLAG peptide were resolved by SDS/PAGE on a 4–12% gradient gel and visualized by silver staining. Specific bands were cut off and subjected to mass spectrometry analysis.

Autophagy Analysis. Autophagy was evaluated under two situations: starvation and rapamycin treatments. For starvation, cells were washed three times with PBS and incubated in EBSS in the presence of E64d (2 μg/mL) and pepstatin (2 μg/mL) for 2–4 h at 37 °C. For rapamycin treatment, cells were incubated in

complete medium containing 500 nM rapamycin (Sigma) for 12 h. Autophagy was assessed by GFP-LC3 redistribution and LC3 conjugation. For GFP-LC3 redistribution, U_2OS cells were transfected with a GFP-LC3 expression plasmid. At 36 h after transfection, GFP-LC3 was detected under normal and rapamycin treatment conditions by using a fluorescence microscope (Zeiss AxioImager 373). The percentage of GFP-LC3-positive cells with punctuate staining was determined in three independent experiments. To quantify GFP-LC3-positive autophagosomes per transfected cell, 5 random fields representing 200 cells were counted. For the detection of endogenous LC3 conjugation, cells with different treatments were sonicated in 1× SDS loading buffer. Cell lysates were heated and then subjected to immunoblotting analysis with an antibody against LC3 (Sigma).

Bacterial Infection Assay. *Atg7*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and its wild-type cell strain were kind gifts from Masaaki Komatsu at Department of Biochemistry, Juntendo University School of Medicine, Tokyo, Japan. *Salmonella typhimurium* SL1344 carrying an EGFP was a kind gift from Denise Monack at the Department of Microbiology and Immunology, Stanford University School of Medicine. Cells were maintained in DMEM (GIBCO) supplemented with 10% FBS. Barkor-inducible knockdown U_2OS were induced with 0.5 μg/mL DOX for 48 h before the infection assay was performed. *Atg7*$^{-/-}$ or control wild-type MEFs and Barkor WT and knockdown cells were seeded 12 h before infection in 6-well culture dishes containing 5 12-mm-diameter round glass coverslips (10^5 cells per well). *S. typhimurium* infections were performed as described below. Briefly, *S. typhimurium* SL1344 carrying an EGFP expression plasmid was grown overnight in LB broth containing 100 mg/L carbenicillin at 37 °C with aeration and then was subcultured at a dilution of 1:33 for a further 3 h in LB. This culture was further diluted in DMEM 10% FCS without antibiotics, to yield a multiplicity of infection of 100. Infections were allowed to proceed for 30 min. Cells were then washed with PBS and incubated in fresh complete medium containing 100 μg/mL gentamicin sulfate for different times as indicated. The number of bacteria per coverslip was determined at the time points indicated in each figure by separately placing coverslips, in triplicate, into 15-mL conical tubes containing 5 mL of sterile water. After a 15-min incubation at room temperature and subsequent vigorous vortexing, serial dilutions were plated onto the LB agar plates, which were incubated at 37 °C for 20 h, after which the colony number was calculated. For microscope analysis, infected cells were fixed in 4% formalin in PBS for 15 min, washed in PBS, and stained with the anti-mouse tubulin antibody (first antibody) and rhodamine red-conjugated anti-mouse IgG secondary antibody.

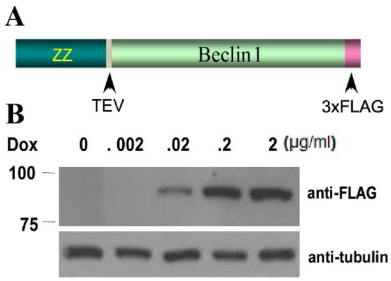

Fig. S1. The inducible expression of ZZ-Beclin 1–3×FLAG. (*A*) Schematic representation of the ZZ-Beclin 1–3×FLAG protein. A tobacco etch virus (TEV) protease cleavage site was inserted between the ZZ domain and Beclin 1 cDNA. The expression of this fusion protein is under the control of a DOX-inducible promoter. (*B*) Western blot analysis of cell extracts from a stable cell line induced by different doses of DOX with an anti-FLAG antibody. Anti-tubulin antibody was used as a control.

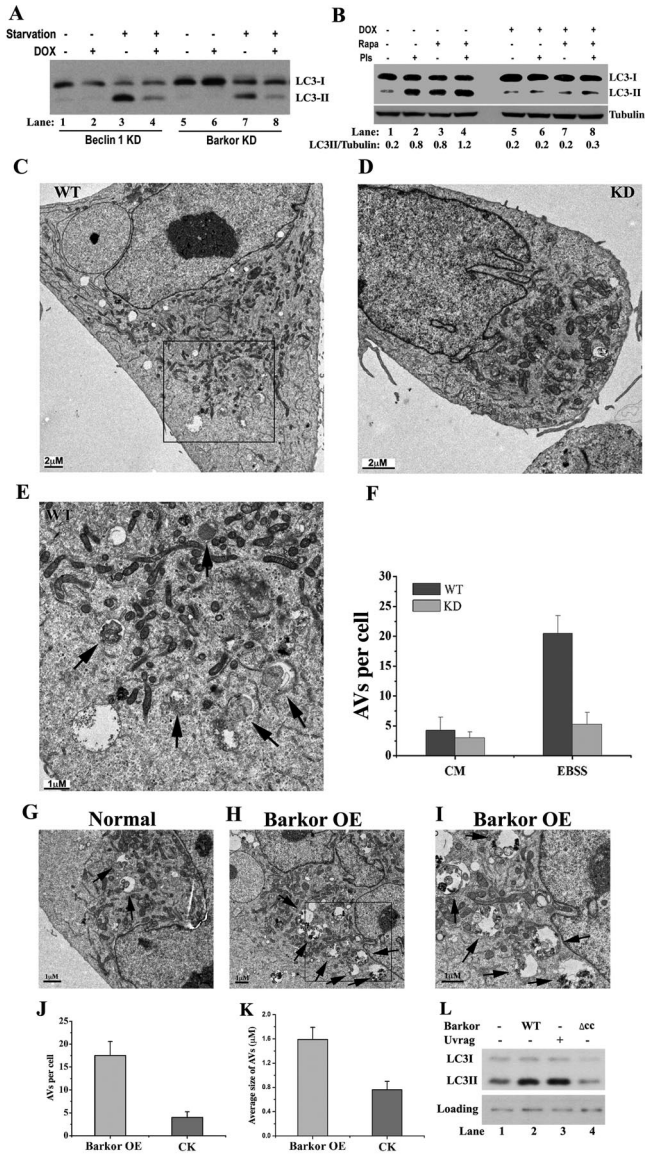

Fig. S2. Protein sequence analysis: Barkor was identified as a novel protein. Peptide hits for mass spectrometry were matched perfectly to the KIAA0831 protein sequence. Peptide hits of Barkor are highlighted in red. A zinc finger motif is underlined (blue); the coiled-coil domain (CCD) is underlined (black).

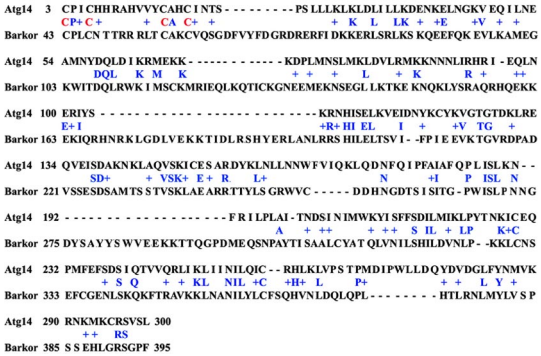

Fig. S3. Alignment of human Barkor with yeast Atg14. The identical and similar residues are marked in blue, and a shared Zinc finger motif is marked in red. Barkor shares 18% sequence identity and 32% sequence similarity with yeast Atg14.

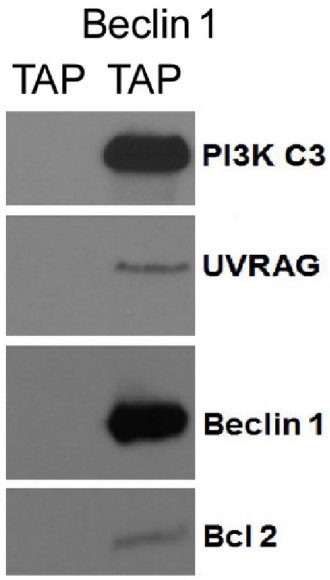

Fig. S4. Western blot analysis of tandem affinity purification. The final eluates of tandem affinity purification of Beclin 1 complex or vector alone (Fig. 1*A*) were analyzed by immunoblotting with anti-PI3K, anti-UVRAG, anti-Beclin 1, and anti-Bcl-2 antibodies.

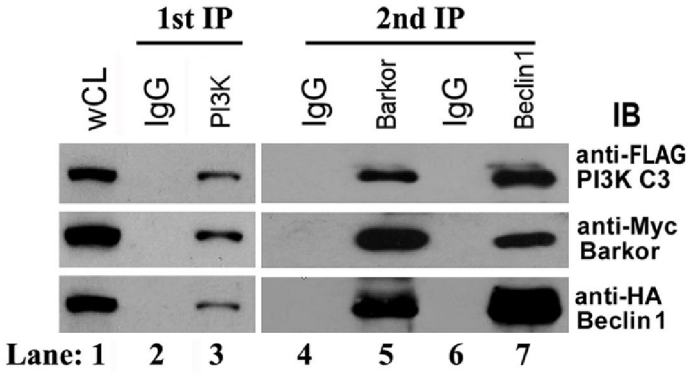

Fig. S5. Barkor is a component of PI3K class III complex. 293T cells were transfected with Myc-Barkor, HA-Beclin 1, and FLAG-PI3KC3. Forty-eight hours after transfection, WCL was immunoprecipitated first with anti-FLAG Ab (PI3KC3) or control IgG, and the FLAG peptide eluate was then immunoprecipitated with either anti-Myc (Barkor) or HA (Beclin 1) Abs. The immunoprecipitates were analyzed by immunoblotting with anti-Myc, HA, or FLAG antibodies.

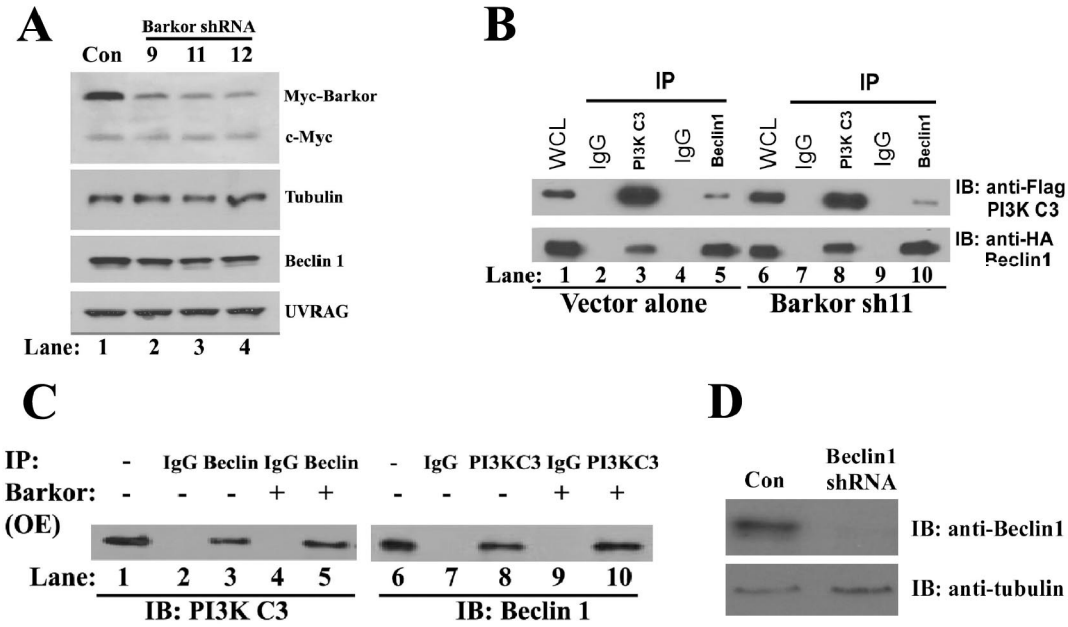

Fig. S6. Analysis of the interaction between Beclin 1 and PI3K in different settings. (*A*) Establishment of Barkor RNAi knockdown stable clones in 293T cells. Control cells and knockdown cells were transfected with Myc-Barkor. Protein levels of Barkor, c-Myc, tubulin, Beclin 1, and UVRAG were detected by Western blot analysis. (*B*) Beclin 1–PI3KC3 complex formation in Barkor-knockdown cells. Barkor-knockdown 293T cells or control cells were transfected with FLAG-PI3KC3 and HA-Beclin 1. Forty-eight hours after transfection, WCLs were immunoprecipitated with anti-FLAG (PI3KC3) or HA (Beclin 1), and the immunoprecipitates were analyzed by immunoblotting with anti-HA or FLAG antibodies. (*C*) Beclin 1–PI3KC3 complex formation in the presence of Barkor overexpression (OE). Forty-eight hours after transfection with Myc-Barkor, FLAG-PI3KC3 and HA-Beclin 1, WCLs were immunoprecipitated with anti-FLAG (PI3KC3) or HA (Beclin 1), and the immunoprecipitates were analyzed by immunoblotting with anti-HA or FLAG antibodies. (*D*) Establishment of Beclin 1 RNAi knockdown stable clones in 293T cells. Protein levels of Beclin 1 and tubulin were detected by Western blot analysis.

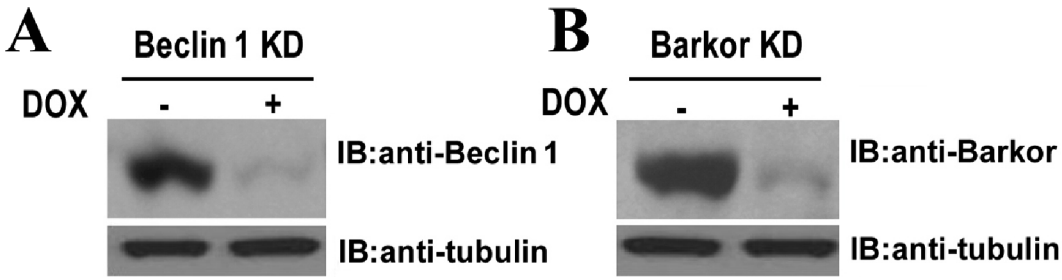

Fig. S7. Inducible knockdown of Beclin1 and Barkor. (*A*) A U_2OS^{TetR} (U_2OS cells stably express Tet repressor) cell line expressing shRNA against Beclin1 was treated with 1 μg/mL DOX. 3 days after induction. WCLs were prepared and analyzed by immunoblotting with anti-Beclin 1 antibody. (*B*) A U_2OS^{TetR} cell line expressing shRNA against Barkor was treated with 1 μg/mL DOX. Three days after induction, WCLs were prepared and analyzed by immunoblotting with anti-Barkor antibody.

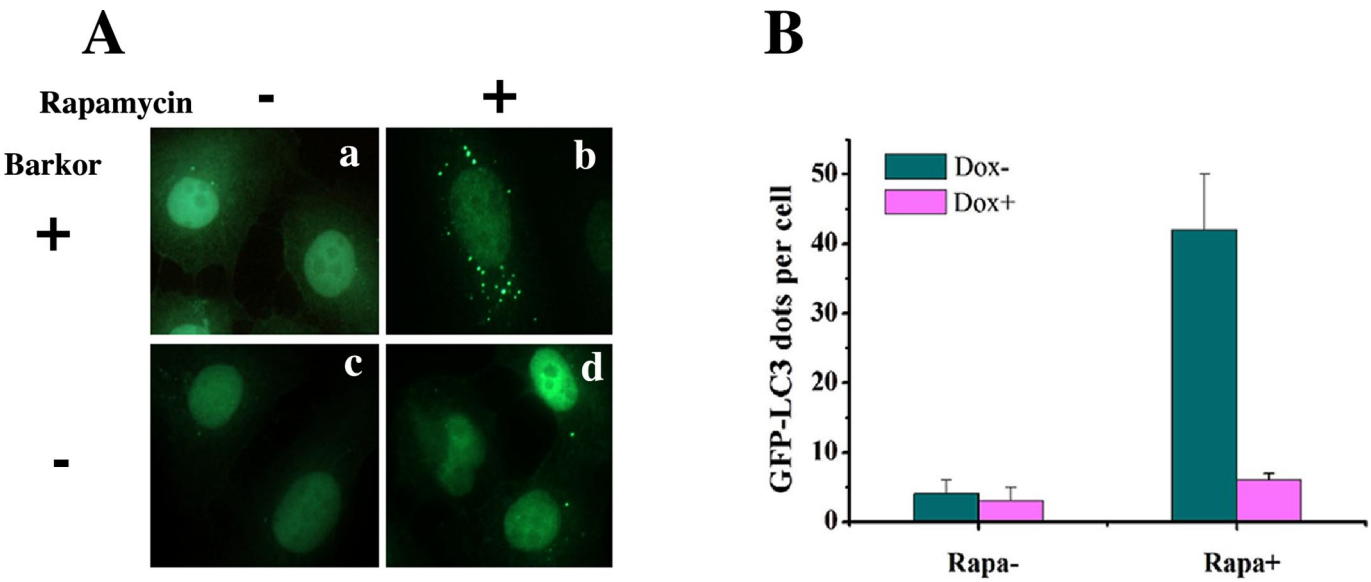

Fig. S8. Autophagosome formation in Barkor-knockdown cells. (*A*) Barkor-knockdown U_2OS cells were transfected with EGFP-LC3 expression vector. Thirty hours after transfection, cells were treated with 2 μM rapamycin for another 4 h. A representative punctate staining of GFP-LC3 is shown. (*B*) Statistical analysis of GFP-LC3 foci per cell was performed and summarized from data from three independent experiments.

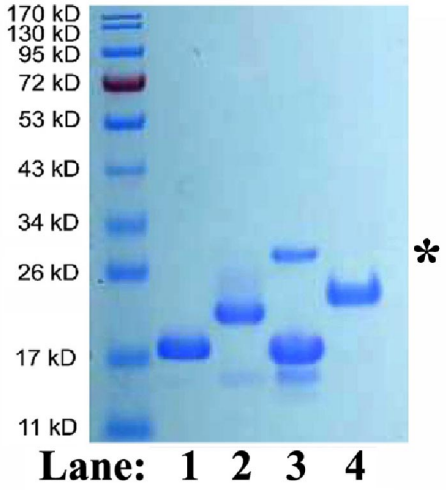

Fig. S9. Purification of recombinant CCD proteins. Recombinant His-Beclin 1-CCD (lane 1), 3×FLAG-Beclin 1-CCD (lane 2), 3×FLAG-UVRAG-CCD (lane 3), and 3×FLAG-Barkor-CCD (lane 4) were expressed and purified from *Escherichia coli* and analyzed by SDS/PAGE and Coomassie blue staining. * indicates a nonspecific protein.

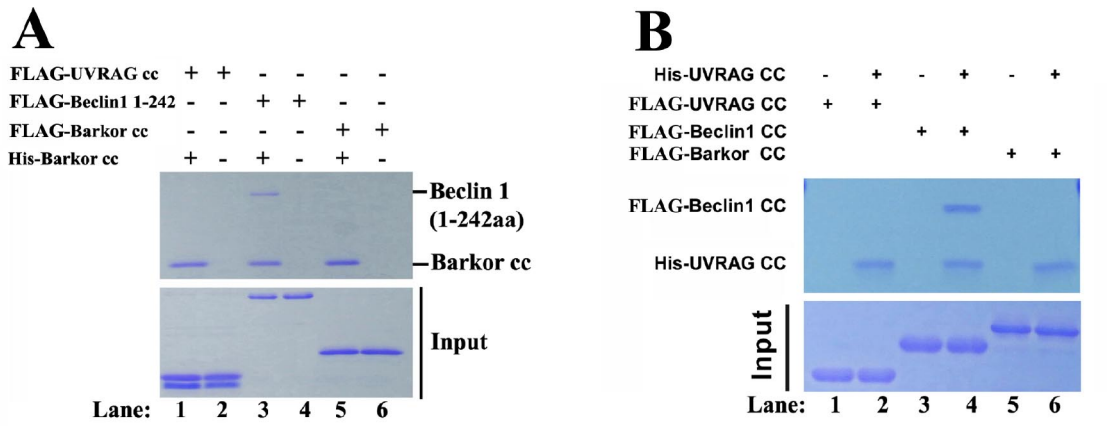

Fig. S10. UVRAG and Barkor form distinct Beclin 1 complexes. (*A*) Direct interaction between Barkor and Beclin 1. Purified recombinant proteins were subjected to an in vitro His_6 pulldown assay. A Ni column was incubated first with His-Barkor CC and then with Flag tagged Beclin 1-(1–242 aa), Barkor-CC, and UVRAG-CC. After extensive washing, proteins bound to beads were analyzed by Coomassie blue staining. (*B*) Direct interaction between UVRAG and Beclin 1. Purified recombinant proteins were subjected to an in vitro His_6 pulldown assay. A Ni column was incubated first with His-UVRAG-CC, and then FLAG-tagged Beclin 1-CC, Barkor-CC, and UVRAG-CC. After extensive washing, proteins bound to beads were analyzed by Coomassie blue staining.