**Figure S1.** Identification of *ATG4BCRISPR* HepG2 cells and sequence homology alignments of Ser34 of ATG4s.(**A**) The genotype of HepG2 cells with or withoutCRISPR/Cas9 targeting was identified by PCR. (**B**) The effectiveness of CRISPR/Cas9 targeting for ATG4B protein level in HepG2 cells was assayed by immunoblotting. (**C and D**) Sequence homology alignments of Ser34 of ATG4s in different species (**C**) and different human ATG4 isoforms (**D**).

**Figure S2.** The Ser34 of ATG4B is phosphorylated by AKT in HCC cells.(**A**) *ATG4BCRISPR* HepG2 cells were transfected with the indicated expression plasmids, and then the cell lysates were subjected to immunoprecipitation and immunoblotting assays. (**B**) The indicated immunogen was prepared and then injected into the skin on the back of New Zealand white rabbits accompanied with Freund's adjuvant. Then the antibody titer of the immune serum was measured with ELISA, taking the non-immune serum as a control. (**C**) *ATG4BCRISPR* HepG2 cells were transfected with ATG4BWT-expressing plasmid, and then the cell lysate was treated with or without lambda phosphatase. Subsequently, immunoblotting assays were performed with the antibody prepared in (**B**) or Flag antibody. (**D**) *ATG4BCRISPR* HepG2 cells were transfected with the indicated expression plasmids, and the cell lysates were subjected to immunoblotting assays with the antibody prepared in (**B**) or Flag antibody. (**E**) HepG2 cells were transfected with the control siRNA or *PTEN* siRNA-1 or *PTEN* siRNA-2, and then the cell lysates were subjected to immunoblotting assays. *PTEN* siRNA-1 resulted in an efficient interference of PTEN and activation of AKT (S473), so it was used for the subsequent experiments. (**F**) *ATG4BCRISPR* HepG2 cells were transfected with the indicated expression plasmids, taking the empty vector (EV) as a control. Then the cell lysates were subjected to immunoblotting assays. MYC-AKT1WT, 1×MYC-tagged wild type AKT1 expression plasmid; 3×Flag-ATG4BWT, 3×Flag-tagged wild type ATG4B expression plasmid; 3×Flag-ATG4BS34A, 3×Flag-tagged mutant ATG4B expression plasmid (in which Ser34 of ATG4B was mutated to Ala); WCL, whole cell lysate; p-ATG4B (S34), Ser34-phosphorylated ATG4B; p-AKT (S473), Ser473-phosphorylated AKT; ATG4BWT, 1×Flag-tagged wild type ATG4B expression plasmid; ATG4BS34A, 1×Flag-tagged mutant ATG4B expression plasmid (in which Ser 34 of ATG4B was mutated to Ala); ATG4AWT, 1×Flag-tagged wild type ATG4A expression plasmid.

**Figure S3.** ATG4A is not phosphorylated by AKT. (**A**) Prediction of the potential phosphorylation sites in human ATG4A and the corresponding upstream kinases using the online website (http://scansite.mit.edu/motifscan\_seq.phtml). (**B**) HepG2 cells were transfected with the control siRNA or *PTEN* siRNA. Then the cell lysates were loaded onto an SDS-PAGE gel with or without Phos-tag acrylamide and MnCl2 for immunoblotting assays. p-ATG4A, phosphorylated ATG4A; non-p-ATG4A, non phosphorylated ATG4A; phos, SDS-PAGE gel containing Phos-tag acrylamide and MnCl2.

**Figure S4.** There is no difference between the effects of ATG4BWT and ATG4BS34A on autophagy in HCC cells.(**A**) *ATG4BCRISPR* HepG2 cells were transfected with the indicated expression plasmids, taking empty vector (EV) as a control. Then the cell lysates were subjected to immunoblotting assays. The corresponding ratios of LC3-II to ACTB are listed below. (**B**) HepG2 cells stably expressing GFP-LC3 were transfected as in (**A**). Then the cells were labeled with the indicated antibody using an immunofluorescence assay and observed under a fluorescence microscope, and the GFP-LC3 puncta in the cells were counted. Representative images are shown (bar: 10 μm). The statistical diagram represents the average number of GFP-LC3 puncta per cell (50 cells per sample were observed). Data are mean±SD from 3 independent experiments. \*\*, *P* < 0.01; ns, no significance. ATG4BWT and ATG4BS34A were the corresponding expression plasmids as described in **Figure S2**.

**Figure S5.** Activation of AKT contributes to the ATG4B-enhanced Warburg effect in HCC cells.(**A and B**) *ATG4BCRISPR* HepG2 cells were cotransfected with the indicated expression plasmids.Then the color of the culture media was observed (**A**), and the concentrations of L-lactate in the culture media were measured (**B**). (**C**) *ATG4BCRISPR* HepG2 cells were cotransfected as in (**B**). Then the concentrations of glucose in the culture media at different time points were detected separately. The time at 6 h after plasmid transfection was considered as the “0 h” time in the diagram. (**D**) *ATG4BCRISPR* HepG2 cells were seeded into 24-well microplates and treated as in (**B**). Then the oligomycin-sensitive respiration (OSR) was examined. Data are mean±SD from 3 independent experiments. ns, no significance. AKT1WT, 1×MYC-tagged wild-type AKT1 expression plasmid; AKT1AA, 1×MYC-tagged mutant AKT1 expression plasmid (in which both Thr308 and Ser473 were mutated to Ala); ATG4BWT and ATG4BS34A were the corresponding expression plasmids as described in **Figure S2**.

**Figure S6.** ATG4B can locate in mitochondria in HCC cells and its mitochondrial translocation is enhanced by AKT overexpression.(**A**) The whole cell lysates (WCL) and mitochondrial fractions were separated from 2 patient-derived HCC tissues (#1 and #2), and then subjected to immunoblotting assays to detect the indicated proteins. The mitochondrial fractions were obtained using a differential gradient centrifugation strategy. (**B**) The mitochondrial fraction was isolated from a patient-derived HCC tissue (#3) using the anti-TOMM22 magnetic beads method. Then the washing buffer that contained mitochondria-free proteins (wash) and elution fraction (mitochondrial fraction) were subjected to immunoblotting assays. (**C**) *ATG4BCRISPR* HepG2 cells were transfected with the indicated expression plasmids. Then the cells were stained with MitoTracker Red (MT-Red) and observed under a fluorescence confocal microscope. The colocalization of GFP-puncta and mitochondria was evaluated. The data in the statistical diagram were from 30 random fields of 3 samples (10 fields per sample). (**D**) *ATG4BCRISPR* HepG2 cells were transfected with the indicated expression plasmids. Then the mitochondrial and cytosol fractions were obtained for immunoblotting assays. Data are mean±SD from 3 independent experiments. ns, no significance. Mito, mitochondria; GFP-ATG4BWT, 1×GFP-tagged wild-type ATG4B expression plasmid; GFP-ATG4BS34A, 1×GFP-tagged mutant ATG4B expression plasmid (in which Ser34 of ATG4B was mutated to Ala); AKT1WT, AKT1AA, ATG4BWT and ATG4BS34A were the corresponding expression plasmids as described in **Figure S5**.

**Figure S7.** ATG4B suppresses the activity of F1Fo-ATP synthase.(**A**) HepG2 cells stably expressing GFP-LC3 were transfected with the indicated siRNAs and expression plasmids. Then the cells were stained with MitoTracker Red (MT-Red) and observed under a fluorescence confocal microscope. The average of GFP-LC3 puncta that colocalized with mitochondira per cell was calculated (n=60 cells/sample from 3 independent experiments). (**B**) HepG2 cells were transfected with control siRNA or *ATG7* siRNA, and then the cells were subjected to immunofluorescence assays with the indicated antibodies. Subsequently, the cells were observed under a fluorescence confocal microscope. Representative images are shown (bar: 10 μm). The statistical diagram represented the percentages of positive cells containing 10 or more red fluorescent puncta (50 red fluorescent cells were observed in one field, n=5). (**C**) *ATG4BCRISPR* HepG2 cells were cotransfected with the GFP-ATG4BWT expression plasmid and *PTEN* siRNA. Then the cells were subjected to immunofluorescence assays with the indicated antibodies. Subsequently, the cells were observed under a fluorescence confocal microscope and the representative images were shown (bar: 10 μm). The colocalization of GFP-puncta and red-fluorescent puncta in 30 random fields from 3 samples was evaluated. (**D**) The lysates from HepG2 or Hep3B cells were separately subjected to immunoprecipitation and immunoblotting assays. (**E**) MgATP submitochondrial particles (SMPs) were separated from a patient-derived HCC tissue (#4) and incubated with or without recombinant human ATG4B protein (rhATG4B) or ATG4A protein (rhATG4A). Then the activity of ATP synthase was detected. Data are mean ± SD from 3 independent experiments. \*, *P* < 0.05; \*\*, *P* < 0.01; ns, no significance. ATG4BWT, ATG4BS34A and GFP-ATG4BWT were the corresponding expression plasmids as described in