Supplementary Methods

Antibodies

The following antibodies were used: anti-actin (Sigma); anti-ALFY rabbit polyclonal antibody (Simonsen A, et al., 2004); anti-ALFY raised in rabbit against recombinant protein corresponding to the N-terminal amino acids (1-330aa); anti-GFP mouse monoclonal antibody (Clontech, 632380); anti-Flag mouse monoclonal (Sigma, F1804); anti-MBP mouse monoclonal antibodies (NEB, e8032s); anti-GABARAP rabbit polyclonal (MBL, PM037); anti-LC3B rabbit polyclonal (MBL, PM036); anti-LC3B rabbit polyclonal (CST, 2775S); anti-GABARAPL1 rabbit polyclonal antibody (Proteintech, 11010-AP); anti-p62 guinea pig polyclonal antibodies (Progen,GP62-C); anti-α-Tubulin (Sigma, T5168); and horseradish peroxidase-conjugated antimouse and anti-rabbit polyclonal antibody (Jackson). The following fluorescent secondary antibodies were used: Cy2-labelled anti-guine pig antibody and Cy3-labelled donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc); Alexa647-labelled anti-mouse antibody (Invitrogen); IRDye680RD donkey anti-rabbit and -mouse antibody and IRDye800 donkey anti-mouse antibody (LICOR).

Cell culture and inhibitors

Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% foetal bovine serum, 5 U/ml penicillin and 50μg/ml streptomycin at 37°C and 5% CO2. FlpIn T-Rex™ HeLa cell line (Invitrogen) with inducible expression of GFP-GABARAP or GFP-LC3B described in [1], were cultured according to the manufacturer’s protocol (Invitrogen). To get desired expression of GFP-GABARAP/-LC3B the cells were treated with 500 ng/ml tetracycline for 24h. Starvation of cells was performed using Earls Balanced Salt Solution (EBSS, Invitrogen). Bafilomycin A1 (AH Diagnostics) was routinely used at 100nM and MG132 (Calbiochem) at 5μM.

Cell transfections

Subconfluent HeLa and U2OS cells were transfected using Lipofectamin 2000 (Invitrogen) and X-tremeGENE 9 (Roche), transfection of HEK293T cells was performed with PEI (Polysciences). GFP-tagged ALFY and its LIR mutant were overproduced in Alfy-knockout MEFs using the helper-dependent adenovirus vectors (HD AdVs). The construction method of the HD AdVs and
the phenotypes of Alfy-deficient mice will be presented elsewhere. siRNA mediated knockdown of ALFY (WDFY3) was performed using a custom made oligonucleotide having the sequence 5`-UAU UCG GCG GAG CAU UUC CUU UU-3` (Dharmacon). For single siRNA transfection cells were routinely transfected with siRNA at a 30 nM concentration using RNAiMAX (Invitrogen) and cells were harvested three days later. Nontargeting siRNA (Dharmacon, D-001810) were used as control. To knock down the entire subfamily of GABARAP proteins a total concentration of 150 nM siRNA was used and cells were harvested after three days. siRNA SMARTpools, consisting of four RNAduplexes, GABARAP (M-012368-01), GABARAPL1 (M-014715-01) or GABARAPL2 (M-006853-02) and non-targeting siRNAs control (D-001206-14) were purchased from Dharmacon. Knockdown was verified by immunoblotting.

**Western blot analysis**

To analyze the cellular levels of different proteins, cells were lysed in RIPA lysis buffer (1% NP40, 0.5% Sodium deoxycholate and 0.1%SDS diluted in PBS + protease inhibitor cocktail). Protein concentrations were determined with BCA Protein assay reagent (Pierce) and approximately 15 μg of protein per sample was loaded and resolved on 4–20% gradient gels (Bio-Rad, Criterion gel), followed by electroblotting to Immobilon-FL membranes (Millipore). The blots were probed with specific antibodies, which were detected using ECL reagents or the Odyssey® Infrared Imaging System (LI-COR).

**Differential detergent extraction**

To determine the cellular levels and solubility of p62, cells were first extracted in ice-cold lysisbuffer (1% Triton X-100, 50mM Tris pH 7.5, 50mM NaCl, 5mM EDTA + protease inhibitor cocktail) , centrifuged at 17000xg for 10 minutes and the supernatants (TX-100 soluble fraction) collected. Pellets were washed with phosphate buffered saline (PBS) before it was solubilized in SDS lysisbuffer (2%SDS, 50mM Tris pH7.5, 1mM EDTA + protease inhibitor cocktail) with sonication (TX-100 insoluble fraction). Protein concentration in both fractions was determined with BCA Protein assay reagent (Pierce) before samples were used for Western blot analysis as described above.

**Immunofluorescence and microscopy**
Cells were fixed in 10% formalin solution, 4%PFA or methanol, permeabilized with 0.05% saponin (Sigma), 50 µg/ml digitonin or methanol, blocked with 0.1% (w/v) gelatin (Sigma) in PBS for 30 min, stained and mounted in prolonged gold (MedProbe). Confocal images were acquired on an Olympus FluoView 1000 laser scanning confocal microscope based on an Olympus IX81 inverted microscope fitted with a Super Apochromat 60X/1.35 oil objective and the following laser lines: UV laser diode (405nm, 6mW), multi-line Ar laser (457nm, 488nm, 515nm, 30mW), Green laser (561nm, 10mW) and Red Helium-Neon laser (633nm, 10mW). Images were acquired using the dedicated FV1000 software. Image processing and analysis were done with OLYMPUS FLUOVIEW Viewer software version 1.7a, ImageJ and Adobe Photoshop CS4 (Adobe Systems, Mountain View, CA).

**In vitro pulldown assays**

Glutathione-S-transferase (GST) and maltose binding protein (MBP) tagged fusion proteins were expressed in *Escherichia coli* BL21 (DE3) Star and purified with Glutathione Sepharose™ (GE Healthcare Bio-Sciences AB) or amylose resin (New England Biolabs), respectively.

*In vitro translation and GST pulldown.* 35S-methionin (PerkinElmer) labeled GFP-tagged proteins were co-transcribed/translated *in vitro* using the TnT T7 coupled reticulocyte lysate system (Promega). The 35S-labeled proteins were mixed with GST-tagged proteins, bound to Glutathione Sepharose, and incubated in NETN-buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 6 mm EDTA, 6 mM EGTA, 0.5% Nonidet P-40, 1 mM dithiothreitol supplemented with Complete EDTA-free protease inhibitor cocktail (Roche Applied Science)) for 1 h at 4°C, washed five times with NETN-buffer, boiled with 2× SDS gel loading buffer, and subjected to SDS-PAGE and transfer onto PVDF membrane. The membranes were analyzed with Ponceau or Coomassie brilliant blue (CBB) staining. 35S-Labeled proteins were detected by a phosphor screen (GE Healthcare) and developed in a Typhoon™ phosphor imaging scanner.

*GST-pulldown of recombinant proteins.* Factor Xa (New England Biolabs) was used to cleave ALFY (aa 2981-3526) off MBP-ALFY (aa 2981-3526) bound to beads. After digestion supernatant was collected and mixed with GST fusion proteins and incubated in NETN-buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 6 mm EDTA, 6 mM EGTA, 0.5% Nonidet P-40, 1 mM dithiothreitol supplemented with Complete EDTA-free protease inhibitor cocktail (Roche
Applied Science) for 1 h at 4°C and washed five times with NETN-buffer. The bound proteins were analyzed by SDS-PAGE followed by Western blotting. The PVDF membrane was stained immunoblotted with anti-MBP and anti-ALFY antibody.

**MBP-pulldown of recombinant proteins.** Precision protease was used to cleave the GST-tag off GST-GABARAP and GST-LC3B. Purified GABARAP/LC3B was analyzed by SDS-PAGE followed by Coomassie brilliant blue (CBB) staining to determine purity. LC3B/GABARAP were then mixed with the different MBP fusion proteins and incubated in TNE-buffer (50 mm Tris–HCl, pH 7.4, 100 mm NaCl, 0.1 mm EDTA) for 1 hr at 4°C before precipitation with amylose resin. The mixtures were washed five times with TNE-buffer. The bound proteins were analyzed by SDS-PAGE followed by Western blotting. The PVDF membrane was stained immunoblotted with anti-LC3B, anti-GABARAP and anti-MBP antibody.

**Strep-pulldown of endogenous GABARAP.** STREP-FLAG tagged proteins were expressed in HEK293T cells. Cells were lysed in TNE buffer (50mM Tris, pH7.5, 150mM NaCl, 1mM EDTA, 1%TX-100 supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics). After 10 min on ice, insoluble proteins were removed by centrifugation at 15,000 rpm for 15 min at 4°C. The resulting supernatants were subjected to affinity purification with Strep-Tactin Sepharose (IBA) according to the manufacturer's instructions. Purified protein complexes were verified by SDS-PAGE followed by immunoblotting.

**GFP-pulldown of endogenous ALFY.** GFP-tagged proteins were either transiently expressed in in HeLa cells or stably expressed in FlpIn T-REx HeLa cells. Cells were lysed in TritonX-100 lysisbuffer (50 mM Tris/Cl pH 8.0, 150 mM NaCl, 1% TritonX-100 supplemented with Complete Protease Inhibitor Cocktail [Roche Diagnostics]). After 30 min on ice with occasional mixing, cell lysates were centrifuged at 10 000xg for 10 min at 4°C. The resulting supernatants were subjected to affinity purification with μMACS epitope tag protein isolation kit according to the manufacturer's instructions (Miltenyi Biotec). Purified protein complexes were verified by SDS-PAGE followed by immunoblotting.

**Isothermal titration calorimetry (ITC)**
ITC was performed at 25°C using a Microcal iTC200 System (GE Healthcare). Experiments were conducted as follows, with all injections performed from a stirring syringe (1000 rpm): aliquots (2 μl) of 1.0 mM ALFY-LIR peptide were injected 20 times at 2.0-min intervals into a sample cell containing 200 μl of 0.1 mM Atg8 homologue proteins. Binding data were analyzed using the computer program Origin, version 7.0, supplied by MicroCal, Inc.

Digital PCR

Using the Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science), cDNA was synthesized from 1 μg of total RNA. Digital PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems) on a BioMark HD System (Fluidigm) according to the manufacturer’s instructions. Specific primers for Atg8 homologs and FAM-labeled PrimeTime assay probes were purchased from Integrated DNA Technologies. TaqMan GAPDH Control Reagents-VIC labeled (Applied Biosystems) were used to quantify a reference gene expression. Data analysis was performed by dPCR analysis software (Fluidigm). The positive numbers of partition in the dPCR panel were counted, and the absolute amounts of target genes were determined.

Protein expression and purification

GABARAP was subcloned into pGEX4T vector, and expressed in *Escherichia coli* BL21. The protein was purified using glutathione-Sepharose 4B, cation exchange, and gel-filtration chromatography. The GST moiety was proteolytically removed by thrombin protease. The purified protein was concentrated to 0.94 mg/ml by ultrafiltration in 25 mM Tris-HCl pH7.5, 1 mM dithiothreitol. A peptide representing residues 3341-3354 from ALFY was added in a 4:1 molar ratio of peptide to GABARAP and incubated for 1 hr at 4 °C.

Crystallization, X-ray data collection, structure determination and refinement
Crystallization of the GABARAP-ALFY peptide complex was performed using hanging drop vapor diffusion method at 293K in drops containing a mixture of 1 μl of protein solution and 1 μl of reservoir solution, which consisted of 2 M ammonium sulfate, 0.1 M sodium citrate pH 5.4 and 0.2 M potassium sodium tartrate. Crystals were equilibrated in cryoprotectant buffer containing reservoir buffer plus 20% (v/v) glycerol. X-ray diffraction data sets for the GABARAP-peptide complex were collected at 100 K on BL44XU at SPring8 (Hyogo Japan). Data processing and reduction were carried out with HKL2000[2]. The crystals belonged to space group $P_{3_2}2_1$, with one molecule in the asymmetric unit. Data collection and refinement statics are summarized in supplementary Table S1 online. The crystal structure of the GABARAP-ALFY peptide complex was solved by molecular replacement using MOLREP[3], with the structure of wild-type GABARAP (PDB ID code 1GNU) as the search model. Models were subsequently improved through alternate cycle of manual rebuilding using COOT[4] and refinement with the program REFMAC5[5]. The final refined model contains residues 1-117 of GABARAP. For the ALFY peptide, the density allowed building 12 residues. Refinement statics are summarized in supplementary Table S1 online. There were no residues in the disallowed regions of the Ramachandran plot. Protein Data Bank accession numbers for the GABARAP-ALFY peptide complex is 3WIM. Structure figures were generated using UCSF Chimera[6] and PyMOL.