SUPPLEMENTARY FIGURES AND FIGURE LEGENDS

A

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Supplementary Figure 1

B

C

D

E

IP: anti-TMEM59
WB: anti-TMEM59

IP: anti-TMEM59
WB: anti-TMEM59

IP: anti-TMEM59
WB: anti-TMEM59

IP: anti-TMEM59
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**Supplementary Figure S1.** (A) Alignment of TMEM59 from different species. References on the left column indicate Uniprot accession numbers of the indicated genus. The boxed regions indicate a putative leader peptide (1-35; Uniprot) and a possible transmembrane stretch (240-262; TMHMM Server) that leaves 61 residues of probable intracellular region. Individual residues highlighted in black and grey indicate aminoacid identity and conservative changes, respectively. The figure shows that TMEM59 is well conserved during evolution. The protein seems to have no obvious orthologs in flies, worms or yeast. (B) Northern-blots of multiple human tissues (left) and cell lines (right) showing ubiquitous expression of TMEM59. Two specific mRNA bands were detected (1.5 and 1.2 kb, as indicated). Size markers for the multiple-tissue blots are shown on the left. Brain presented particularly low, and Saos2 osteosarcoma cells particularly high, TMEM59 mRNA levels. A number of subsequent experiments to characterize the endogenous TMEM59 protein were carried out in Saos2 cells, taking advantage of these increased expression levels. (C) Western-blot analysis of endogenous TMEM59 reveals a ubiquitous 34-36 kD diffuse band. Total protein lysates from the indicated cell lines were subjected to immunoprecipitation using a rabbit anti-human TMEM59 antibody or irrelevant protein-G beads, as indicated. The resulting immunoprecipitates were processed for Western-blotting with a chicken anti-human TMEM59 antibody. This combination of immunoprecipitation plus Western-blot was necessary to detect the endogenous protein. (D) Specificity of the TMEM59 protein band. Saos2 (top panel) or HeLa (bottom panel) cells were transfected with the indicated siRNAs and lysed 48 h later for rabbit anti-TMEM59 immunoprecipitation plus chicken anti-TMEM59 Western-blotting. The figure shows complete depletion of the relevant 34-36 kD band. Two minor, low molecular weight bands in HeLa cells (as indicated) appeared to be also specific. (E) Treatment with tunicamycin produced a
faster migrating and more compact TMEM59 protein band in Western-blots, indicating N-glycosylation. Saos2 and Hela cells (as indicated) were treated for 18 h with 10 μg/ml of tunicamycin (a general N-glycosylation inhibitor), lysed and subjected the anti-TMEM59 immunoprecipitation plus Western-blot scheme that allows detection of the endogenous protein. Asterisks indicate irrelevant bands in D and E.
**Supplementary Figure S2.** Endogenous TMEM59 localizes to small cytoplasmic vesicles and is rapidly degraded in the lysosome. (A) TMEM59 specifically localizes to small cytoplasmic vesicles. Saos2 cells were transfected with the indicated siRNAs and, 48 h later, stained for anti-TMEM59 immunofluorescence. Representative confocal images are shown. White arrows indicate the relevant vesicles. The right panel displays an anti-TMEM59 immunoprecipitation plus anti-TMEM59 Western-blotting assay showing successful depletion of endogenous TMEM59. (B) Ectopic TMEM59 shows a similar subcellular localization. Saos2 cells expressing TMEM59-HA were transfected with the indicated siRNAs and stained for TMEM59 as in A. Representative confocal pictures are shown. The right panel displays an anti-HA Western-blot showing TMEM59-HA depletion. (C) Rapid constitutive degradation of TMEM59. Saos2 cells were treated for the indicated times (minutes) with the protein synthesis inhibitor cycloheximide (CHX, 10 µg/ml) and subjected to anti-TMEM59 immunoprecipitation plus anti-TMEM59 Western-blotting. The figure indicates that endogenous TMEM59 has a short half-life (25-30 min). (D) Lysosomal inhibition induces TMEM59 protein levels. Saos2 cells were treated with the indicated inhibitors (bafilomycin, 50 nM; NH₄Cl, 10 mM; E64d/Pepstatin, 10 µg/ml each) for the shown times, and lysed for TMEM59 immunoprecipitation plus Western-blotting. (E) Lysosomal inhibition increases the number of TMEM59-positive vesicles. Saos2 cells were treated with the lysosomal protease inhibitors E64d and pepstatin (10 µg/ml) for 8 h and processed for anti-TMEM59 immunofluorescence. Representative confocal images are shown. (F) Lysosomal inhibition does not significantly alter TMEM59 mRNA levels. Saos2 cells were treated as in D. Shown are Northern-blots for TMEM59. The lower panels display denaturing agarose gels stained with ethidium bromide prior to Northern-blotting. (G) Autophagy is not involved in the constitutive degradation of TMEM59. Wild-type or
Atg5-deficient MEFs were treated as indicated (bafilomycin 50 nM, 8 h) and lysed for TMEM59 immunoprecipitation plus Western-blotting (top panel), or for Western-blotting against the indicated molecules. (H) Induction of autophagy does not alter TMEM59 expression levels. Cells were starved (Saos2: 8 h; HeLa-TMEM59-HA: 16 h) or treated with rapamycin (2 µg/ml, 8 h), and lysed for TMEM59 immunoprecipitation plus Western-blotting (Saos2 cells, top panel), or for Western-blotting against the indicated molecules. Asterisks indicate irrelevant bands (D, G and H).
**Supplementary Figure S3.** Endogenous TMEM59 mainly localizes to the late endosomal/lysosomal compartment. (A-B) Endogenous TMEM59 strongly colocalizes with the late endosome/lysosome markers LAMP2 and CD63, partially with the early endosome marker EEA1 and does not colocalize with the Golgi marker GM130. Untreated Saos2 cells (A), or Saos2 cells treated with the lysosome protease inhibitors E64d (10 μg/ml) and pepstatin (10 μg/ml) for 8 h (B), were stained for double immunofluorescence against TMEM59 (green) and the indicated subcellular markers (red). Representative confocal images are shown. White arrows indicate examples of the relevant vesicles. (C) Transfected TMEM59 localizes to the cell surface. The indicated cell lines were transfected with empty vector or a plasmid expressing TMEM59 (as shown) and, 36 h later, stained before permeabilization with anti-TMEM59 antibodies to detect the presence of TMEM59 at the cell surface. Representative confocal pictures are shown. The bright field pictures confirm that the TMEM59 signal actually localizes to the cell surface. (D) Endogenous TMEM59 localizes to the cell surface after treatment with chloroquine. Saos2 cells were left untreated or treated with chloroquine (24 h, 50 μM), as indicated, and stained for surface TMEM59 as in C. Representative confocal images are shown. (E) Quantification of the phenotype shown in D. Mounted preparations were scored for the percentage of cells showing TMEM59 surface signal. Scoring was done by blindly counting at least ten different fields (around 400 cells) using a conventional microscope. Data are expressed as the mean -/+ s.d. of one representative experiment of three repetitions. TMEM59 was completely absent from the cell surface in basal conditions. (F) Specificity of the TMEM59 cell surface signal. Saos2 cells were transfected with the indicated siRNAs and, 48 h later, treated with chloroquine (24 h, 50 μM), stained for surface TMEM59 and scored as in E. This figure confirms that the scored signal is specific. The right panel displays a combination of
anti-TMEM59 immunoprecipitation plus anti-TMEM59 Western-blotting showing successful depletion of endogenous TMEM59. The asterisk indicates an irrelevant band.
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Supplementary Figure 4

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CD16:7
siRNA
-/+ anti-CD16 Ab
HA-LC3A-I
HA-LC3A-II
ACTIN

ATG5

B

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CD16:7
siRNA
-/+ anti-CD16 Ab
HA-LC3A-I
HA-LC3A-II
ACTIN

ATG7

C

% of cells showing aggregated GFP-LC3A

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CD16:7
Supplementary Figure S4. The minimal active subdomain of TMEM59 promotes LC3 activation through an autophagic pathway that relies on ATG5 and ATG7. (A, B) 293 cells were transfected with the indicated siRNAs and, 10-12 h later, transfected for a second time with the shown CD16:7 chimeras mixed with plasmids expressing HA-LC3A and GST. Cells were then subjected to aggregation with anti-CD16 antibodies and lysed for Western-blotting against the indicated proteins. Panels on the right display control Western-blots of lysates from unaggregated cells showing successful depletion of the relevant protein as well as equal loading (ACTIN), transfection (GST) and comparable chimera expression (CD16). (C) ATG5 is necessary for GFP-LC3A activation by the active subdomain of TMEM59. 293 cells were transfected with the indicated siRNAs and, 10-12 h later, with the shown CD16:7 chimeras mixed with a plasmid expressing GFP-LC3A. Cells were subjected to chimera aggregation and mounted for quantification of activated GFP-LC3A. Quantification was carried out by establishing the percentage of transfected cells showing redistributed GFP-LC3A. At least ten different fields were counted per experimental point (about 400 cells). The experiment was repeated three times. Data are expressed as means +/- s.d. of the triplicates. Results are consistent with the data from the HA-LC3A conversion experiments (A).
## Supplementary Figure 5

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HEK-293 TMEM59 overexpression

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HEK-293 CD16:7-chimera aggregation

### C

C

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### D

D

[Images of CD16:7 localization and colocalization with green vesicles]
Supplementary Figure S5. (A) Overexpressed TMEM59 colocalizes with activated GFP-LC3. 293 cells were transfected with full-length TMEM59 or a deleted version lacking the intracellular domain (ΔID), plus vectors encoding GFP-LC3A (left) or GFP-LC3B (right), and stained 36 h later with anti-TMEM59 antibodies (red). Representative confocal pictures are shown. (B) Aggregated, endocytosed CD16:7 chimeras containing the whole intracellular domain of TMEM59 (ID) or just the active subdomain (263-281), colocalize with activated GFP-LC3. 293 cells were transfected with the indicated CD16:7 chimeras and vectors expressing GFP-LC3A (left) or GFP-LC3B (right), subjected to aggregation with anti-CD16 antibodies and stained for the chimeric construct (red). Representative confocal images are shown. (C) Quantification of vesicles that contain aggregated, endocytosed CD16:7-263-281 chimeras and colocalize with activated GFP-LC3 in JAR cells. JAR cells were transfected with the CD16:7-263-281 chimera and a vector expressing GFP-LC3A, aggregated with anti-CD16 antibodies and stained for the endocytosed chimera (red). Preparations were scored by blindly counting the number of red vesicles colocalizing with green ones. At least fifty cells were scored per experimental point. The experiment was repeated three times. Data are expressed as means +/- s.d. of the triplicates. The figure shows that the vast majority of vesicles containing endocytosed chimera were labeled with GFP-LC3A. (D) Overexpressed TMEM59 localizes to single membrane vesicles. 293 cells were transfected with a plasmid expressing TMEM59-HA and, 36 h post-transfection, fixed and processed for anti-HA immunoelectron microscopy (12 nm gold particles). The figure shows three representative examples of the typical vesicles generated by expression of the molecule. Untransfected cells showed complete absence of these vesicles. All vesicles containing anti-HA signal presented single membranes. Arrows
indicate single membrane (black) or anti-HA signal (white). Scale bar: 400 nm for micrograph #1 and 200 nm for micrographs #2 and #3.
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Supplementary Figure 6

A

Time after transfection (h)

24 36

Vector TMEM59 Vector TMEM59

p62-HA

TMEM59

AU-LC3A-I

AU-LC3A-II

ACTIN

B

263-281

CD16:7

Control - +

CD16:7 anti-CD16 Ab

Vector Vector

p62-HA

TMEM59

AU-LC3A-I

AU-LC3A-II

ACTIN

C

Balkomkon Starvation

Control - +

p62-HA

TMEM59

AU-LC3A-I

AU-LC3A-II

ACTIN

D

Time after transfection (h)

24 36 48 62

Vector TMEM59 Vector TMEM59

p62-HA

TMEM59

AU-LC3A-I

AU-LC3A-II

ACTIN

E

263-281

CD16:7

Control - +

CD16:7 anti-CD16 Ab

Vector Vector

p62-HA

TMEM59

AU-LC3A-I

AU-LC3A-II

ACTIN

F

Balkomkon Landseer Starvation

Control - +

p62-HA

TMEM59

AU-LC3A-I

AU-LC3A-II

ACTIN

UB

75

50

G

24 36 48 62

Vector TMEM59 Vector TMEM59

HA-NBR1

TMEM59

AU-LC3A-I

AU-LC3A-II

ACTIN

H

263-281

CD16:7

Control - +

CD16:7 anti-CD16 Ab

Vector Vector

HA-NBR1

TMEM59

AU-LC3A-I

AU-LC3A-II

ACTIN

I

Balkomkon Landseer Starvation

Control - +

HA-NBR1

TMEM59

AU-LC3A-I

AU-LC3A-II

ACTIN

UB

J

% of transfected cells with aggregated GFP-mt-Q7A

Transfection TMEM59 Starb. Mab

Control n.s.

***

ns

n.s.

n.s.
Supplementary Figure S6. The autophagic activity induced by TMEM59 does not influence the expression levels of conventional autophagic substrates. (A, D, G) TMEM59 overexpression does not alter the levels of cotransfected p62-HA or HA-NBR1. 293 cells were transfected with TMEM59 or empty vector (as shown), AU-LC3A and p62-HA (A, D) or HA-NBR1 (G), and lysed after the displayed times in an NP40-based lysis buffer (A) or a 2x SDS lysis buffer that solubilizes protein precipitates (D, G). Shown are Western-blots against the indicated molecules. The figures show that TMEM59 expression does not alter the levels of cotransfected p62-HA or HA-NBR1 irrespective of the lysis method used. (B, E, H) Aggregation of the CD16:7-263-281 chimera does not modulate p62-HA or HA-NBR1. 293 cells were transfected with the indicated chimera, AU-LC3A and p62-HA (B, E) or HA-NBR1 (H), subjected to aggregation with anti-CD16 antibodies and lysed in NP40 (B) or 2x SDS (E, H). Shown are Western-blots. (C, F, I) Autophagy modulators alter the levels of ectopic p62-HA and HA-NBR1. 293 cells were transfected with AU-LC3A and p62-HA (C, F) or HA-NBR1 (I) and, 24 h post-transfection, treated as shown (bafilomycin, 200 nM, 16 h; lactacystin (a proteasome inhibitor), 10 µM, 16 h; starvation, 8 h). Cells were lysed in NP40 (C) or 2x SDS (F, I) for Western-blotting. Anti-ubiquitin (UB) control blots (F, I) show accumulation of ubiquitinated substrates in the presence of lactacystin, demonstrating that the drug did inhibit the proteasome. Figures show that p62-HA and HA-NBR1 are degraded mainly by autophagy and only partially through the proteasome (lactacystin). (J) TMEM59 overexpression does not influence the clearance of aggregated GFP-huntingtin-Q74 (GFP-htt-Q74). JAR cells were transfected with GFP-htt-Q74 and TMEM59 or empty vector (as shown), treated 24 h later as indicated (starvation, 20 h; bafilomycin, 200 nM, 16 h; treated samples were transfected with control vector) and mounted for microscopy. Samples were quantified
for the percentage of transfected cells showing intracellular GFP-positive aggregates. At least ten different fields were counted (about 300 cells). The experiment was repeated three times. Data are expressed as means +/- s.d. of the triplicates. (n.s.), no significant differences (P > 0.5, paired Student’s t-test); asterisks, significant differences (P < 0.01). The figure shows that TMEM59 does not alter the number of GFP-htt-Q74 precipitates, whereas starvation and bafilomycin have the expected effect.
Supplementary Figure S7. TMEM59 does not mediate the autophagic degradation of conventional autophagic substrates. (A) Depletion of TMEM59 with specific siRNAs does not alter the autophagic modulation of endogenous p62 or LC3 in three different cell lines. The indicated cells (bottom) were transfected with the indicated siRNAs and, 48 h later, treated with the shown modulators of autophagy (top panels: bafilomycin (Baf., 100 nM, 16 h); middle panels: starvation (Starv., 8 h)). Cells were subsequently lysed in an NP40-based lysis buffer for Western-blotting against the indicated molecules. The bottom panels display a combination of anti-TMEM59 immunoprecipitation plus anti-TMEM59 Western-blotting showing successful depletion of endogenous TMEM59. (B) Depletion of TMEM59 in HeLa cells does not alter the autophagic modulation of p62 or NBR1, whether these are endogenous or ectopically expressed molecules, and whether the cells are lysed in an NP40-based lysis buffer, or in the 2x SDS lysis buffer that provides solubilization of protein precipitates. The indicated HeLa cell strains (bottom) were transfected with the indicated siRNAs and treated as in A, lysed in the indicated buffers (bottom) and processed for Western-blotting against the indicated molecules (HA-tagged molecules detected with an anti-HA antibody). The lowest panels show TMEM59 immunoprecipitation assays of parallel NP40 lysates demonstrating successful depletion of the molecule. This figure shows that TMEM59 depletion does not alter in any way the levels of p62 or NBR1 in any of the tested experimental settings. Treatment with bafilomycin induced accumulation of higher molecular weight NBR1 forms that were also unaffected by depletion of TMEM59.
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Supplementary Figure 8

A

% of transfected cells with aggregated GFP-miQ74

Control    TMEM59    Control    TMEM59    Control    TMEM59

- Starvation  Baflomycin  ➤ Treatment

siRNA

B

C    T ➤ siRNA

C : Control siRNA
T : TMEM59 siRNA

IP: anti-TMEM59
WB: anti-TMEM59
**Supplementary Figure S8.** TMEM59 does not mediate autophagic degradation of GFP-huntingtin-Q74 (GFP-htt-Q74). (A) JAR cells were transfected with the shown siRNAs and, 36 h later, transfected again with a construct expressing GFP-htt-Q74. Cells were treated 24 h later with the indicated modulators of autophagy (starvation, 20 h; bafilomycin, 16 h, 200 nM), and then mounted for microscopy. Samples were quantified for the percentage of transfected cells showing intracellular GFP-positive aggregates (most cells contained just one GFP-positive precipitate). Counts and statistics were done as in **S6J**. The figure shows that absence of TMEM59 does not alter the modulation of GFP-htt-Q74 aggregates provided by regulators of conventional autophagy, like starvation and bafilomycin. The bottom panel (B) displays a combination of anti-TMEM59 immunoprecipitation plus anti-TMEM59 Western-blotting showing successful depletion of endogenous TMEM59.
Supplementary Figure 9
**Supplementary Figure S9.** Reduced degradation of the CD16:7-263-281 chimera in the presence of lysosomal inhibitors. 293 cells were transfected with the indicated CD16:7 constructs, aggregated in the absence or presence (as shown) of the lysosomal inhibitors E64d (10 µg/ml) and pepstatin (10 µg/ml), and lysed for Western-blotting. The figure shows overexposed anti-CD16 Western-blots.
Supplementary Figure S10. (A) Comparable cell surface expression levels of the CD16:7-263-281 mutants to alanine utilized in the alanine scanning analysis. 293 cells were transfected with the indicated constructs and, 36 h later, processed for anti-CD16 flow cytometry. The graph displays percentages of positive cells (left axis) and means of fluorescence of positive cells (MF, right axis) obtained from triplicates. Data are expressed as means +/- s.d. of the triplicates. (B) Simultaneous mutation to alanine of the four essential aminoacids in the active subdomain reduces the ability of TMEM59 to promote HA-LC3 conversion by overexpression. 293 cells were transfected with wild-type or mutated (4M, quadruple mutant) TMEM59 versions in a full-length (FL) or Δ282 (largest functional deletion) background (as indicated), along with HA-LC3A and GST. Cells were lysed 24 h later for Western-blotting against the indicated molecules. (C) Simultaneous mutation of the four essential aminoacids blocks the ability of overexpressed TMEM59 to promote GFP-LC3 activation and colocalization with TMEM59-positive vesicles. 293 cells were transfected with the indicated TMEM59 versions along with GFP-LC3A, and stained 36 h later with anti-TMEM59 antibodies (red). Representative confocal images are shown. (D) Cell surface levels of the CD16:7-263-281 quadruple mutant (4M) compared to the wild-type construct. 293 cells were transfected and, 36 h later, processed for anti-CD16 flow cytometry. Data are expressed as in A. The figure shows slightly higher surface expression of the mutant construct. (E) Number of endocytic vesicles generated by CD16:7-263-281 chimeras containing a wild-type active subdomain or the 4M version. JAR cells were transfected with the indicated chimeras, subjected to anti-CD16 aggregation and stained for the endocytosed construct. Cells were scored by blindly counting the number of chimera-positive vesicles per cell. At least fifty cells were scored per experimental point. The experiment was repeated three times. Data are expressed as means +/- s.d. of the triplicates. The
figure shows decreased numbers of endocytic vesicles containing the mutant construct. (F) Endocytosed CD16:7-263-281-4M colocalizes with EEA1 and CD63 in JAR cells. Procedures were as in Figure 4B. Representative confocal pictures are shown. See results with the CD16:7-263-281 wild-type version (Figure 4B) for comparison.
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Supplementary Figure 11

A

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B

% of positive cells

MF of positive cells

CD16.7-263-281 mutants
**Supplementary Figure S11.** (A) Functional analysis of CD16:7-263-281 mutant constructs where the four essential residues of the active subdomain were individually subjected to conservative mutations. 293 cells were transfected with the indicated CD16:7-263-281 mutants and a plasmid expressing HA-LC3A, subjected to aggregation with anti-CD16 antibodies and lysed for Western-blotting against the indicated molecules. (B) Cell surface expression levels of the mutant CD16:7-263-281 constructs used in A. 293 cells were transfected with the indicated constructs and, 36 h later, processed for anti-CD16 flow cytometry. The graph displays percentages of positive cells (left axis) and means of fluorescence of positive cells (MF, right axis) obtained from triplicates. The data are expressed as mean values +/- s.d. of the triplicates. The figure shows comparable expression levels of all relevant constructs.
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Supplementary Figure 12

+ untagged ATG16L1

- untagged ATG16L1
**Supplementary Figure S12.** Frequent apposition events between GFP-ATG5 and endocytosed CD16:7-263-281 in the presence of untagged ATG16L1. JAR cells were transfected with CD16:7-263-281 and GFP-ATG5, with or without a plasmid expressing untagged ATG16L1 (as indicated), aggregated for 4 h and stained for the endocytosed chimera (red). Representative confocal pictures are shown. Examples of two different cells are provided in both cases. The figure shows that GFP-ATG5 mainly displays a diffuse intracellular distribution (with few discrete events) in the absence of ATG16L1, and therefore it is difficult to evaluate colocalization with the endocytosed chimera in these conditions. In the presence of cotransfected ATG16L1, most of the GFP-ATG5 signal translocates to discrete structures that frequently localize in close contact with the endocytosed chimera.
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Supplementary Figure 13

A

Control 263-281
Contr. ATG16 Contr. ATG16

CD16.7

siRNA

1/4 anti-CD16 Ab

HA-LC3A-I

HA-LC3A-II

ACTIN

Unaggregated samples

B

GFP-LC3-positive vesicles (CD16.7/263-281)

siRNA

Control ATG16

Control ATG16

60 80 100

0 20 40 60

% of CD16.7-positive vesicles colocalizing with green vesicles

Control 263-281
Supplementary Figure S13. Critical role of ATG16L1 for the ability of the active subdomain to promote LC3 activation. (A) 293 cells were transfected with the indicated siRNAs twice in the same day (first transfection in the morning and second transfection 10-12 h later), split after an overnight incubation and transfected the next day with the shown CD16:7 chimeras mixed with a plasmid expressing HA-LC3A. Cells were then subjected to aggregation with anti-CD16 antibodies and lysed for Western-blotting against the indicated proteins. Panels on the right display control Western-blots of lysates from unaggregated cells showing successful depletion of endogenous ATG16L1 as well as equal loading (ACTIN) and comparable chimera expression (CD16). (B) JAR cells were transfected twice with the indicated siRNAs (as in A), split and transfected the next day with the shown CD16:7 chimeras mixed with a plasmid expressing GFP-LC3A. Cells were then aggregated and stained for the endocytosed chimera (red). Preparations were scored by blindly counting the number of red vesicles (chimera) and green vesicles (GFP-LC3A) per cell, as well as the number of red vesicles colocalizing with green ones. At least fifty cells were scored per experimental point. The experiment was repeated three times. The graph shows the number of GFP-LC3A vesicles per cell expressed as the percentage of the value obtained for the active chimera (CD16:7-263-281) in the sample transfected with control siRNAs (left axis), and the percentage of chimera vesicles labeled with GFP-LC3A (right axis). Data are expressed as means +/- s.d. of the triplicates. Asterisks indicate significant differences (paired Student’s t-test; P < 0.01). Panels on the right display control Western-blots of lysates from unaggregated cells showing successful depletion of endogenous ATG16L1 as well as equal loading (ACTIN) and comparable chimera expression (CD16).
Supplementary Figure S14. (A) The ATG16L1-binding motif present in TMEM59 interacts with the sorting adaptor AP1M1. 293T cells were transfected with the indicated constructs, lysed and subjected to GST immunoprecipitation using agarose beads coupled to glutathione (IP, immunoprecipitation; WB, Western-blot; TL, total lysate). Shown are Western-blots against the indicated molecules. AP1M1 was expressed untagged. (B) The ATG16L1-binding motif interacts with the sorting adaptor AP2M1. Procedures were as in A. The asterisk indicates an irrelevant band that is also present in untransfected cells. (C) Interaction between TMEM59 and ATG16L1 is not mediated by the sorting adaptors AP1/AP2 or clathrin. 293T cells were transfected with the indicated siRNAs twice in the same day (first transfection in the morning and second transfection 10-12 h later), split after an overnight incubation and transfected the next day with the indicated DNA constructs. Cells were lysed 36 h post-transfection and subjected to GST immunoprecipitation using agarose beads coupled to glutathione. Shown are Western-blots against the indicated molecules. The bottom panels display control Western-blots of total cellular lysates showing successful depletion of the relevant proteins. (D) Simultaneous mutation of Y277 and L280 to alanine (2M) in 263-281 inhibits binding to AU-ATG16L1 in a pull-down assay. An anti-ATG16L1 antibody was used to detect AU-ATG16L1. Procedures were as in Figure 6E.
Supplementary Figure S15. (A) The WD domain of ATG16L1 is necessary and sufficient for the interaction between NOD2-CARD1 and ATG16L1. 293T cells were transfected with the indicated constructs, lysed and subjected to GST immunoprecipitation using agarose beads coupled to glutathione (IP, immunoprecipitation; WB, Western-blot; TL, total lysate). Shown are Western-blots against the indicated molecules. (B) The WD domain of ATG16L1 is necessary and sufficient for the interaction between TLR2-ID and ATG16L1. Procedures were as in A.
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Supplementary Figure 16

A

\[
\begin{align*}
\text{TMEM59-WT} & \quad \cdots \text{CATVA TAVEQ} \quad \underline{\text{YVPSEKLSIYGDL}} \quad \text{FMNEQ} \cdots \\
\text{TMEM59-Δ268} & \quad \cdots \text{CATVA TAVEQ} \\
\text{TMEM59-Δ282} & \quad \cdots \text{CATVA TAVEQ} \quad \underline{\text{YVPSEKLSIYGDL}} \\
\text{TMEM59-Δ268-NOD2 (63-76)} & \quad \cdots \text{CATVA TAVEQ} \quad \underline{\text{WEVLSWEDYGFHLLG}} \\
\text{TMEM59-Δ268-TLR2 (761-779)} & \quad \cdots \text{CATVA TAVEQ} \quad \underline{\text{YLEWPMDEAQREGFWVNLR}} \\
\text{TMEM59-Δ268-DEDD2 (12-25)} & \quad \cdots \text{CATVA TAVEQ} \quad \underline{\text{WEDEECLYYGMLS}} \\
\text{TMEM59-Δ268-T3JAM (518-533)} & \quad \cdots \text{CATVA TAVEQ} \quad \underline{\text{WRSQYEALKEDWRTL}}
\end{align*}
\]

B

\[
\begin{align*}
\text{TMEM59-Δ268-} & \\
\text{TMEM59-construct} & \quad \underline{\text{A268} \quad \underline{\text{A262} \quad \underline{\text{NOD2 (63-76)} \quad \underline{\text{TLR2 (761-779)} \quad \underline{\text{DEDD2 (12-25)} \quad \underline{\text{T3JAM (518-533)}}}}}}
\end{align*}
\]

IP: GSH
WB: anti-TMEM59
IP: GSH
WB: anti-GST
TL: anti-TMEM59
TL: anti-GST
TL: anti-ACTIN
**Supplementary Figure S16.** The minimal peptides containing the ATG16L1-binding motif in NOD2-CARD1 (aminoacids 63-78), TLR2-ID (761-779), DEDD2 (12-25) and T3JAM (318-333) suffice to coimmunoprecipitate with GST-ATG16L1 when fused to a deleted version of TMEM59 lacking the intracellular region. (A) Aminoacid sequence of the intracellular tail of all TMEM59 derivatives used in the experiment. The sequence of TMEM59-WT is provided as a reference and the boxed region indicates the minimal 19-aminoacid peptide containing the ATG16L1-binding motif. A deleted version of TMEM59 lacking all aminoacids beyond the first residue of the motif (TMEM59-Δ268) was used both as a negative control for immunoprecipitation and also as the template used to swap the relevant peptides; this construct contains just five intracellular aminoacids (TAVEQ). The largest TMEM59 C-terminal deletion that remains active and includes the whole active peptide in TMEM59 (TMEM59-Δ282) was used as a positive control. (B) 293T cells were transfected with the indicated constructs, lysed and subjected to GST immunoprecipitation using agarose beads coupled to glutathione (IP, immunoprecipitation; WB, Western-blot; TL, total lysate). Shown are Western-blots against the indicated molecules. The figure shows that all constructs coimmunoprecipitate with GST-ATG16L1 except the deleted version of TMEM59 lacking the active region (TMEM59-Δ268).
**Supplementary Figure S17.** (A) TMEM59 depletion does not inhibit LC3II generation by SA at late infection times. HeLa cells were transfected with the indicated siRNAs and, 48 h later, infected for 16 h and lysed for Western-blotting. This experiment was carried out in parallel with those shown in Figure 9A. (B) Depletion of TMEM59 with alternative siRNA species inhibits LC3II generation by SA at early infection times. HeLa cells were transfected with the indicated siRNAs and, 48 h later, infected with the bacteria (2 h, moi = 10) before lysis for Western-blotting. The bottom panel shows TMEM59 depletion (rabbit anti-TMEM59 immunoprecipitation plus chicken anti-TMEM59 Western-blot). (C) SA does not alter the levels of p62 or NBR1 at early infection times after cell lysis in SDS. HeLa cells were infected for the shown times (moi = 10) and lysed in an SDS-based lysis buffer for Western-blotting. The figure confirms that the inability of SA to alter the levels of endogenous p62 or NBR1 after NP40 lysis (see Figure 9A) is not due to poor protein solubilization. (D) LC3II generation by SA at early infection times is independent of BECLIN-1 and VPS34. HeLa cells were transfected with the indicated siRNAs and, 48 h later, infected with SA (2 h, moi = 10) before lysis for Western-blotting. The asterisk indicates an irrelevant band. (E) SA is enclosed in LC3-positive, single membrane phagosomes at early infection times. HeLa cells expressing HA-LC3A were infected with SA (2 h, moi = 10) and fixed for anti-HA immunoelectron microscopy (12 nm gold particles). Shown are two representative examples of a common phenotype, as most bacteria were enclosed in single-membrane vesicles and these phagosomes often displayed varying degrees of anti-HA labeling. The engulfed bacteria showed unspecific staining probably due to expression of protein A. Arrows indicate single membrane (black) or HA-LC3A (white). Scale bar: 400 nm. (F) SA induces TMEM59 expression without mRNA contribution. Hela cells were infected with SA (2 h, moi = 10) and lysed for rabbit anti-
TMEM59 immunoprecipitation plus chicken anti-TMEM59 Western-blots. The right panel displays semi-quantitative RT-PCR assays with two different TMEM59 amplicons and an internal control (ACTIN).
SUPPLEMENTARY MATERIALS AND METHODS

Cell culture and reagents

Cell lines were obtained from the ATCC. *Atg5* /-/- MEFs were kindly provided by Dr. N. Mizushima. Cells were cultured at 37º C and a humidified 5% CO₂ atmosphere, in DMEM containing 10% heat-inactivated FBS and 100 U/ml of penicillin/streptomycin (Invitrogen). Cycloheximide, chloroquine, E64d, pepstatin, bafilomycin, rapamycin, lactacylsitin and tunicamycin were from Sigma. Starvation was done in Earle’s Balanced Salt Solution (Sigma). Antibodies: ACTIN, TUBULIN (mAbs, Sigma), GAPDH (mAb, Abcam), ATG5 (rabbit polyclonal, Sigma), ATG7 (rabbit polyclonal, Cell Signaling), LC3, ATG16L1 (mAbs, MBL), Clathrin Heavy Chain, BECLIN-1, p62 (mAbs, BD), Ubiquitin (Enzo), AP1M1 (rabbit polyclonal, Abcam), AP2M1 (mAb, BD), NBR1, VPS34 (rabbit mAbs, Cell Signaling), AU1 (rabbit polyclonal, Covance), HA (mAb, Covance), GST (mAb, Santa Cruz), LAMP2, CD63 (mAbs, University of Iowa Hybridoma Bank), EEA1, EEA1-FITC, CD63-FITC, GM130 (mAbs, BD), CD16 for chimera aggregation (mAb, NA/LE, BD), CD16 for Western-blot (mAb, Santa Cruz), CD16-FITC for flow cytometry (mAb, BD), HRP-coupled goat anti-mouse and anti-rabbit Igs (Dako), polyclonal rabbit anti-mouse IgG for chimera aggregation, goat anti-mouse kappa (unlabeled and Cy3-coupled), HRP-coupled goat anti-chicken IgY, Cy3-coupled goat anti-mouse and anti-rabbit Igs, Cy5-coupled goat anti-mouse Igs (Jackson Immunoresearch), Alexa-488-coupled goat anti-mouse and anti-rabbit Igs (Invitrogen).

Anti-TMEM59 antibodies

Anti-TMEM59 antibodies were generated against a fusion protein between GST and aminoacids 36-239 of TMEM59 expressed in bacteria (pGEX plasmid, GE Healthcare).
Solubility of this protein was improved by lysing bacterial cells in the presence of N-laurylsarcosine, as described (Frangioni and Neel, 1993). Antibodies were generated in rabbit and chicken, and extracted from egg yolks using the Eggcellent IgY purification kit (Pierce). Immunoreactive sera were passed through a column containing immobilized GST (Pierce) to remove GST-specific antibodies, and affinity purified against immobilized GST-TMEM59-36-239. Despite both purification steps, the sera still recognize GST to some extent, particularly when the latter is overexpressed or concentrated by previous immunoprecipitation (see source data for Western-blots presented in Figures 6C, 7B, 7D and 7F). Rabbit antibodies were used for most applications. Chicken antibodies were only used for Western-blotting after immunoprecipitation.

**Origin and manipulation of cDNA constructs**

TMEM59 and bNIP3L were cloned as cell death-inducing molecules from a human cDNA expression library (Alcalá et al., 2008). The p35 expression plasmid was previously described (Klee and Pimentel-Muiños, 2005). The p62-HA construct was obtained from Addgene. LC3A (NM_032514), LC3B (NM_022818), TLR2-ID (NM_003264), T3JAM (NM_025228), DEDD2 (NM_133328), AP1M1 (NM_001130524) and AP2M1 (NM_004068) were cloned by PCR from a human cDNA library. AP1M1 and AP2M1 were expressed untagged. The human NOD1 and NOD2, ATG16L1β, ATG5 and BECLIN cDNAs, and the CD16:7 cassette, were kindly provided by Drs. G. Núñez (University of Michigan Medical School, USA), T. Yoshimori (Research Institute for Microbial Diseases, Osaka, Japan), N. Mizushima (Tokyo Medical and Dental University, Tokyo, Japan), B. Levine (Southwestern Medical Center, Texas, USA) and B. Seed (Harvard Medical School, Boston, USA),
respectively. Plasmids expressing HA-NBR1 and GFP-huntingtin-Q74 were kind gifts from Drs. I. Dikic (Buchmann Institute for Molecular Life Sciences, Frankfurt, Germany) and D. Rubinsztein (Cambridge Institute for Medical Research, Cambridge, UK), respectively. Deletions, fusions and CD16:7 chimeric constructs involving these cDNAs were generated by PCR. Constructs were cloned into the pEAK series of mammalian expression plasmids (that contain the SV40 large T-antigen origin of replication), or a retroviral derivative designated P12-MMP (Klee et al., 2009). All constructs were verified by sequencing.

**Transfections and retroviral transductions**

Transfections were done using the JetPEI lipid reagent (Polyplus). Cells were analyzed 36 h after transfection in overexpression experiments, unless otherwise stated. Cell death caused by TMEM59 overexpression in 293T cells, although obvious with positive identification methods, was never higher than 10-15%, did not have an impact on the amount of the different proteins recovered from the lysates, and was negligible in cells lacking the SV40 large T antigen. Cells stably expressing TMEM59-HA (Saos2 and HeLa), HA-LC3, TMEM59-HA plus Cherry-LC3A or Cherry-ATG16L1 (HeLa) were generated by retroviral transduction with derivatives of the P12-MMP vector including IRES-puromycin (HA-LC3, TMEM59-HA) or IRES-hygromycin (Cherry fusions) resistance cassettes that allow selection in puromycin (1 µg/ml) and hygromycin (200 µg/ml). Retroviral methods were previously described (Klee et al., 2009).

**Western-blotting and immunoprecipitation**

Cells were lysed in a buffer containing 1% Igepal CA-630 detergent (NP40, Sigma), 150 mM NaCl, 50 mM Tris pH 7.5, 5 mM EDTA, protease inhibitor cocktail (Sigma),
PMSF (1 mM), NaF (5 mM) and orthovanadate (200 nM). Lysis in 2x SDS lysis buffer was done by resuspending the cell pellet in a 2x standard sample buffer containing 4% SDS but lacking β-mercaptoethanol and bromophenol blue, followed by extensive boiling. β-mercaptoethanol and bromophenol blue were added to the samples after measurement of protein concentrations. Equal protein amounts were resolved by SDS-PAGE, transferred to a PVDF membrane (Millipore) and probed with specific antibodies. HA-LC3 was always detected using anti-HA antibodies. Blots were developed by chemiluminescence using the ECL system (Amersham).

To immunoprecipitate endogenous TMEM59, total NP40 lysates were incubated with the rabbit anti-TMEM59 antibody (overnight, 4ºC, rotation) and for 1 h with protein G-coupled agarose beads (GE Healthcare). Immunoprecipitates were washed with lysis buffer and subjected to Western-blotting with the chicken anti-TMEM59 antibody. For co-precipitations between endogenous TMEM59 and ATG16L1 during S. aureus infection, total lysates were subjected to anti-TMEM59 immunoprecipitation for 4 h. For co-immunoprecipitations with GST-fusion constructs, total lysates were diluted to reach a final detergent concentration of 0.2%, immunoprecipitated (4ºC, 1-3 h, rotation) using agarose beads coupled to glutathione (GE Healthcare), and washed with immunoprecipitation buffer. To coprecipitate GST-263-281 and ATG16L1, cell lysis and immunoprecipitation were done in a buffer containing 1% CHAPS (Sigma).

**Northern-blotting**

Multiple human tissue Northern-blots were from Clontech. Total RNA was isolated using the RNeasy kit (Qiagen), resolved by denaturing agarose electrophoresis and transferred to a charged nylon membrane (Nytran, Whatman). A 675 bp TMEM59
cDNA fragment between an internal BsrG1 site and the end of the coding sequence was used as a probe. Radioactive labeling was done by random priming (Stratagene).

**siRNA studies**

Cells were transfected with pre-designed pools of siRNA duplexes (On-TargetPlus, Dharmacon) against TMEM59, ATG5, ATG7, ATG16L1, Clathrin Heavy Chain, AP1M1, AP2M1, BECLIN or VPS34, using the transfection reagent DharmaFECT1 (Dharmacon). Individual siRNAs against TMEM59 were from Dharmacon (#7: ggaauugacuuuaucgaa; #8: gaagaggaguuguacgcau). Control siRNAs were a mix of duplexes having no perfect match with any human gene (siControl, Dharmacon).

**Electron microscopy**

Cells were fixed with 2% paraformaldehyde/0.1% glutaraldehyde and mounted in 12% gelatine/2.3% sucrose for ultrathin cryosectioning. Preparations coexpressing aggregated CD16:7-263-281 chimera and hIgG-LC3A were stained with a goat anti-rabbit IgG serum coupled to 18 nm colloidal gold particles (to detect the rabbit anti-mouse antibody that super-aggregates the chimera) and a goat anti-human IgG serum coupled to 12 nm gold particles (to detect hIgG-LC3A). Preparations containing HA-tagged constructs were stained with an anti-HA mAb plus a goat anti-rabbit IgG serum coupled to 12 nm colloidal gold particles. Colloidal gold-coupled secondary reagents were from Jackson Immunoresearch. Samples containing engulfed SA were previously blocked with an irrelevant rabbit pre-immune serum in order to reduce background bacterial staining probably due to protein A expression by these bacteria. Samples were observed under a JEM-1010 electron microscope (Jeol).
Semi-quantitative PCR

Total RNA was isolated with the RNeasy kit (Qiagen) and reverse-transcribed with the SuperscriptII First Strand synthesis system (Invitrogen). PCR conditions were: 95°C, 1 min; 65°C, 1 min; 72°C, 1 min; for 22 cycles. Oligonucleotides were: TMEM59 #1: fw, ccttgacacacctaccta, rev, tgtctcagtcaagcaatgg; TMEM59 #2: fw, ccatgtgtaactgagaca, rev, tgtagccctgctcataat; Actin: fw, agatcaagatcattgctctctg, rev, ctggtttctgcgcaagttgt. Fw and rev primers anneal in different exons.

Flow cytometry

Transfected 293 cells were analyzed 36 h post-transfection. Cells were resuspended in PBS/2% BSA/0.1% azide, incubated for 1 h with an anti-CD16 mAb coupled to FITC (BD) and analyzed using a FACSCalibur cytometer (BD).

Pull-down assays

IPTG-induced bacteria (BL21) expressing GST fusion proteins (pGEX plasmid, GE Healthcare) were lysed by treatment with lysozyme (100 µg/ml), freeze-thawing and sonication. Lysates were cleared by centrifugation and then incubated (1 h, 4°C, rotation) with agarose beads coupled to glutathione (GE Healthcare). Beads loaded with the purified proteins were used for ATG16L1 pull-down from induced crude bacterial lysates. ATG16L1 was expressed from the pET plasmid (Novagen) in BL21(DE3) bacteria. Expression of this construct in the soluble fraction was poor, but detectable. Beads were incubated with the amount of ATG16L1-containing lysate equivalent to 1 ml of induced culture (3 h, 4°C, rotation), washed and processed for Western-blotting.
SUPPLEMENTARY REFERENCES


