Supporting Information

Lassen et al. 10.1073/pnas.1407001111

SI Materials and Methods

Mice. ***T300A knock-in mice.*** In brief, we generated a targeting construct with 5′ and 3′ homology arms of 5.2 and 5.3 kb length, respectively, with a FRT-flanked neomycin resistance cassette between the two homology arms. The T300A polymorphism was introduced by site-directed mutagenesis and was confirmed by sequencing. Following electroporation into BS20 ES cells (Brian Seed 20; derived from 129S1/SvImJ mice), neomycin-resistant colonies were tested for homologous integration. Positive clones were further tested by Southern blotting to verify single homologous recombination. Injected clones underwent germ-line transmission. Homozygous T300A knock-in mice were crossbred with homozygous 129-*FLP*e mice, resulting in removal of the neomycin resistance cassette, and were subsequently crossbred into the C57BL/6 background by using a SNP-based approach (Charles River Laboratories).

Atg16L1 KO and Atg16L1$^{f/f}$ × Villin-cre mice. Mouse embryonic stem cells with validated targeting of the autophagy related 16-like 1 (*Atg16L1*) locus [exon 3 flanked by loxP sites; Atg16L1$^{flox/flox\ (f/f)}$ mice] were purchased from EUCOMM (www.knockoutmouse.org/martsearch/project/23950) as previously described (1). A reporter/resistance cassette composed of an FRT-flanked LacZ/neomycin sequence followed by a LoxP site was placed upstream of the targeted exon. An additional LoxP site was inserted downstream of the targeted exon. Exclusive splicing of the LacZ/neomycin cassette resulted in a reporter KO mouse (Atg16L1 KO). Villin-*cre* mice (stock 004586) were purchased from Jackson Laboratory and bred to Atg16L1$^{f/f}$ mice. Bedding was mixed and littermates were used to minimize variability in microbiome composition. Animals were used at 6–8 wk of age and sex-matched for independent experiments. Animals were housed in a pathogen-free facility, and all procedures were performed in accordance with the institutional animal care and use committee at Massachusetts General Hospital.

Flow Cytometry and Isolation of Intestinal Stem Cells and Paneth Cells. Small intestines were removed and the fat/mesentery was dissected away. The intestinal lumen was washed with cold PBS solution using an 18-gauge feeding needle (FN-7905; Roboz) and opened longitudinally. Mucus was removed by gently rubbing the intestine between fingers in cold PBS solution. Intestines were cut into 3–5-mm fragments and placed into cold PBS solution/10 mM EDTA. Samples were incubated on ice and shaken intermittently for 30 min; supernatant was discarded at least three times. Fragments were resuspended with PBS solution/EDTA and shaken on ice for 10 min; supernatant was discarded three times. Fragments were resuspended with PBS solution/EDTA and incubated on ice and shaken intermittently for 20–40 min. Samples were then triturated with a 10-mL pipette one or two times, and the contents were filtered twice through a 70-μm mesh (BD Falcon) to remove villous material and tissue fragments. At this point, the suspension was mainly composed of crypts. Crypts were removed from this step for crypt culture experiments and embedded in Matrigel with crypt culture media. For intestinal stem cell (ISC) isolation, crypt suspensions were centrifuged for 5 min at 250 × *g* at 4 °C or room temperature. Pellets were gently resuspended in 1 mL of TrypLE Express (Invitrogen) and 120 μL of DNase I (10 U/μL; Roche). Samples were incubated at 32 °C for 1.25–2 min and then placed on ice. Twelve milliliters of cold suspension minimal essential medium (SMEM) was added to each sample, and samples were gently triturated twice. Samples were centrifuged for 5 min at 250 × *g*. Pellets were resuspended and incubated for 15 min on ice in 0.5–1 mL SMEM that contained an antibody mixture consisting of CD45-PE (no. 30-F11; eBioscience), CD31-PE (Mec13.3; BioLegend), Ter119-PE (Ter119; BioLegend), CD24-Pacific Blue (M1/69; BioLegend), and EPCAM-APC (G8.8; eBioscience). SMEM (12 mL) was added, and the samples were centrifuged for 5 min at 250 × *g*. Pellets were resuspended with 0.5–2 mL of SMEM/7-amino-actinomycin D (7-ADD) solution. Samples were filtered through a 40-μm mesh (BD Falcon) before cell sorting. ISCs were isolated as Lgr5-EGFP^{hi}Epcam^{+}CD24$^{low/-}$CD31^{-}Ter119^{-}CD45^{-}7-AAD^{-}; EGFP^{low} progenitors were isolated as EGFP^{low}Epcam^{+}CD24$^{low/-}$CD31^{-}Ter119^{-}CD45^{-}7-AAD^{-}, and Paneth cells were isolated as CD24^{hi}SideScatter^{hi}Lgr5-EGFP^{-}Epcam^{+}CD31^{-}Ter119^{-}CD45^{-}7-AAD^{-} with a BD FACS Aria II SORP cell sorter into supplemented crypt culture medium. Dead cells were excluded from the analysis with the viability dye 7-AAD.

Antibacterial Autophagy Assays. For siRNA experiments, HeLa cells (parental strain or stably transduced with LC3-GFP) were reverse-transfected with 5 pmol siRNA in 96-well glass-bottomed plates at a density of 6×10^3 cells per well for imaging analysis or transfected with 20 pmol siRNA in 12-well plates at a density of 6×10^4 cells per well for quantitative PCR (2). Autophagy assays were performed at 48 h after transfection and treated for the indicated times. Knockdown of individual genes was confirmed by quantitative RT-PCR; Table S1 lists primers. For LC3 co-localization, cells were infected with *Salmonella* as previously described (3). At 1 h after infection, HeLa-LC3-GFP cells were fixed in 2% (wt/vol) paraformaldehyde overnight and stained with Hoechst to visualize nuclei. Fixed cells were imaged in 96-well glass-bottomed plates by using an ImageXpress Micro Widefield High Content Screening System with a 20× lens. LC3-*Salmonella* colocalization was quantified by using Cell Profiler software. Three wells were measured per condition, six sites were measured per well, and each site included ∼75–100 cells, yielding an estimated average of 1,350–1,800 cells counted per condition. Gentamicin protection assays were performed as described previously in HeLa cells (4). Gentamicin protection assays in mouse embryonic fibroblasts (MEFs) were performed by using WT *Shigella* or *Shigella* ΔicsB transformed with pXEN13 to express bacterial luciferase (Perkin-Elmer). MEFs were infected for 30 min, washed twice with PBS solution, and incubated with Iscove's Modified Dulbecco's Medium (IMDM) containing 50 μg/mL gentamicin for an additional 1.5 h. Cells were then washed twice with PBS solution and incubated in IMDM containing 20 μg/mL gentamicin. Plates were read on a TopCount NXT at indicated times. For primary cell replication experiments with *Shigella*, primary cultures of T300A and control small intestinal epithelial cells were grown on transwells as previously described (5). Both apical and basolateral surfaces of the transwell were infected with 1:100 dilution of *Shigella flexneri* ΔicsB growing in stationary phase for 2 h. Transepithelial electrical resistance was monitored every hour. Serial dilutions of cell lysates were prepared at various time points and plated on lysogeny broth plates in triplicate to determine cfu counts. cfu counts were normalized to number of cells on transwells.

Histology and Pathology Scoring. Tissues were fixed in 10% (wt/vol) buffered formalin and embedded in paraffin. Sections (5 μm) were cut and stained with H&E. Longitudinal sections were scored in a blinded fashion by using previously established methods (6). The following parameters were assessed: (*i*) submucosal edema

(scored 0–3), (*ii*) polymorphonuclear leukocyte infiltration into the lamina propria (scored 0–4), (*iii*) goblet cell number (scored 0–3), and (*iv*) epithelial integrity (scored 0–3); the maximum score is therefore 13. Each mouse was scored independently, and histological scores shown were averaged per genotype.

Culture of Isolated Cells in Supplemented Crypt Culture Medium. Isolated ISCs or Paneth cells were centrifuged for 5 min at 250 × *g* and resuspended in the appropriate volume of crypt culture medium (500–1,000 cells per microliter) supplemented with 1× N2 (Invitrogen), 1× B27 (Invitrogen), 100 ng/mL Wnt-3A (R&D Systems), and 500 ng/mL R-Spondin to yield 1 μg/mL final concentration. ISCs were seeded into Matrigel (no. 356231, growth factor reduced; BD Bioscience) containing 1 μM Jagged (AnaSpec) up to 5,000–10,000 cells/30–50 μL. Drops (30 μL) of 65% (wt/vol) Matrigel were plated onto a flat-bottom 48-well plate (no. 3548; Corning), and Paneth cells were added at the same cell count to the top of the Matrigel drop. Alternatively, ISCs and Paneth cells were mixed after sorting in a 1:1 ratio, centrifuged, and then seeded into Matrigel. Matrigel drops with ISCs and Paneth cells were allowed to solidify for 20–30 min at 37 °C. A total of 350 μL of crypt culture medium was then overlaid onto Matrigel drops and maintained at 37 °C in fully humidified chambers containing 6% CO_2. Crypt media was changed every second day. Organoid bodies were quantified on days 3, 7, and 9 d of culture unless otherwise specified.

IL-1β Production and shRNA Infection. IL-1β was detected by sandwich ELISA per manufacturer's protocol (BD Biosciences). Samples were quantified using the SpectraMax M5 microplate reader (Molecular Devices), measuring absorbance at 450 nm. Samples were run in triplicate. Lentiviral shRNA was provided by the RNAi platform at the Broad Institute. A total of 33 genes were screened, with each gene having five individual hairpins. shRNAs targeting IL-1β, caspase 1, Atg5, and Atg16L1 were used as positive controls; luciferase, LacZ, GFP, P2tG-nullT, and empty T shRNA were used as negative controls. Immortalized bone marrow-derived macrophages (BMDMs) were plated in DMEM with 10% (vol/vol) FBS and antibiotics at 1×10^4 in 96-well plates. Lentiviral supernatant (50 μL) was added to each well, and cells were incubated at 37 °C for 3 d. A total of 100 μL fresh media with puromycin (final concentration, 3 μg/mL) was added. After 48 h, puromycin was washed from the cells and fresh media was added. Cells were stimulated with IFN-γ (100 ng/mL), LPS (100 ng/mL), and muramyl dipeptide (10 μg/mL) for 24 h, and supernatants were analyzed by ELISA for IL-1β (BD Biosciences). Cell viability was measured by using Alamar blue (Life Technologies). The screen was repeated in triplicate. Genes were scored based on effect size from the positive and negative controls. Genes that scored ≥100% of the positive control were considered to play a functional role in IL-1β secretion.

Quantitative Real-Time PCR. RNA was extracted from siRNA-treated or shRNA-treated cells at indicated times using the RNeasy Kit (Qiagen). Reverse transcription was performed by using the iScript cDNA Synthesis kit (Bio-Rad Laboratories), and quantitative PCR was performed with SYBR Green Supermix (Life Technologies) using the iCycler with iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Reaction conditions consisted of 40 cycles of PCR with 58 °C or 59 °C annealing temperatures.

Immunoprecipitation and On-Bead Digestion for Proteomic Analysis. Atg16L1$^{-/-}$ MEFs were transfected in 10-cm dishes with empty vector, ATG16L1 isoform 1 (NM_030803.6), ATG16L1 isoform 2 (NM_017974.3), or ATG16L1 T300A (isoform 1 SNP). Cells were lysed in lysis buffer containing 1% Nonidet P-40. Approximately 40 mg of total protein from each sample were immunoprecipitated with 50 μL of magnetic FLAG beads (M2, Sigma). Complexes were eluted two times with 100 μg peptide in 500 μL lysis buffer and affinity-purified with 25 μL Strep magnetic beads. Immunoprecipitation eluates were denatured on beads with 2 M urea/50 mM Trizma and then predigested with sequencing-grade modified trypsin (V5113; Promega) in an enzyme-to-substrate ratio of 1:50 for 30 min at room temperature with on a shaker. The supernatant was collected, and protein disulfide bonds of the combined lysates were reduced for 45 min with 5 mM DTT (no. 20291; Thermo Scientific). Cysteines were subsequently alkylated for 45 min with 10 mM iodoacetamide. Samples were digested overnight at room temperature with trypsin in a 1:50 enzyme-to-substrate ratio on a shaker. Peptide mixtures were acidified to a final volumetric concentration of 1% trifluoroacetic acid. Tryptic peptides were desalted on reversed phase tC18 SepPak columns (WAT036790; Waters), and eluted peptide samples were evaporated to dryness in a vacuum concentrator.

iTRAQ Labeling of Peptides. Desalted peptides were labeled with iTRAQ (isobaric tags for relative and absolute quantification) reagents according to the manufacturer's instructions (AB Sciex) with iTRAQ tags as follows: 114, empty vector; 115, WT isoform 1; 116, T300A; and 117, WT isoform 2. Peptides were dissolved in 0.5 M triethylammonium bicarbonate, pH 8.5, solution, and labeling reagent was added in 380 μL ethanol. After 1 h incubation, the reaction was stopped with 1 M Tris·HCl, pH 7.5. Differentially labeled peptides were mixed and subsequently desalted by using StageTips. Desalted peptides were separated by pH cuts using combined strong cation exchange/reversed-phase stage tips. Samples were dried down by vacuum centrifugation and reconstituted in 10 μL 3% (vol/vol) acetonitrile/0.1% formic acid.

Liquid Chromatography/Tandem MS Analysis. All peptide samples were analyzed by nanoflow liquid chromatography/MS with a Proxeon nanoflow UPLC system coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific). Peptides were resolved on a capillary column (Picofrit with 10-μm tip opening/75-μm diameter; PF360-75–10-N-5; New Objective) packed in-house with 20 cm C18 silica material (1.9 μm ReproSil-Pur C18-AQ medium; Dr. Maisch) with an analytical flow rate of 200 nL/min and a 120-min gradient from 0.1% formic acid in water to 0.1% formic acid/90% (vol/vol) acetonitrile. The run time was 155 min for a single sample, including sample loading and column reconditioning. An MS method with a full scan (70,000 resolution) and data-dependent LTQ MS/MS scans for the top 12 precursors from the MS1 scan was used.

Quantification and Identification of Peptides and Proteins. Peptide quantification and protein identification were performed by using the Spectrum Mill software package (Agilent Technologies). Each MS/MS spectrum was searched against a UniProt mouse protein database with appended contaminants. Protein relative quantification using iTRAQ was performed on the MS/MS scans, represented by the ratio of peak areas of masses of the tags that correspond to the iTRAQ reagents. By dividing the peak areas observed at 115, 116, and 117 *m*/*z* by that at 114 *m*/*z*, the relative amount of a peptide in each sample was calculated. The ratios were corrected for overlapping isotopic contributions, estimating the relative abundances of a particular peptide. Corrected $\log_2$ ratios for bait/vector are reported.

Immune Cell Isolations. Single-cell suspensions were obtained from spleen and mesenteric lymph node by mashing the organs, passing over a 70-μm filter, and lysing red blood cells. Colonic tissues were harvested and placed in ice-cold HBSS. After removal of residual mesenteric fat tissue, colons were opened longitudinally. Tissues were then washed in ice-cold HBSS and cut into 1-cm pieces. After three additional washes in HBSS/1% FBS, tissues were incu-

bated with stir bars in serum-free media with 5 mmol/L EDTA and 0.145 mg/mL DTT at 37 °C and 250 rpm for 30 min. After washing, colon pieces were incubated with stir bars in HBSS/FCS/EDTA for 15 min at 37 °C. Again, pieces were washed with HBSS and set at room temperature for 10 min in HBSS/FCS/15 mM Hepes. Colon pieces were next digested in serum-free media containing 0.16 mg/mL Liberase and 0.1 mg/mL DNase I at 37 °C at 250 rpm for 40 min with stir bars. Cells were washed, passed through a 70-μm cell strainer, and resuspended in 4.5 mL of 44% (wt/vol) Percoll and placed on 2.3 mL of 67% (wt/vol) Percoll. Percoll gradient separation was performed by centrifugation for 20 min at 600 × *g* at room temperature. Lymphoid fractions were collected at the interphase of the Percoll gradient, washed once, and resuspended in MACS buffer. Cells were used immediately for magnetic separation.

CD11c$^+$ and CD11b$^+$ Cell Isolations. Single-cell suspensions from spleen, mesenteric lymph node, and colonic lamina propria were used to isolate CD11c-positive and CD11b-positive populations. Cells were subjected to magnetic positive selection by using MACS technology per the manufacturer's protocol (Miltenyi Biotec).

Ex Vivo CD11c$^+$ Cell Stimulation for IL-1β Production. Cells (2×10^5) were plated in 96-well plates. As indicated, cells were treated with 10 μg/mL LPS for 24 h at 37 °C in 5% CO_2. Supernatants were harvested and frozen at −20 °C before ELISA analysis.

Ex Vivo CD11b$^+$ Cell Stimulation for IL-1β Production. Cells (1×10^5) were plated in 96-well plates and incubated for 1 h at 37 °C in 5% CO_2, at which point the cells had adhered to the plate. All cells were treated with 100 ng/mL IFN-γ. As indicated, cells were treated with 10 μg/mL muramyl dipeptide and 2 ng/mL LPS for 24 h at 37 °C in 5% CO_2. Supernatants were harvested and frozen at −20 °C before ELISA analysis.

Viability Assay. Metabolic activity of splenic macrophages was detected by using Presto Blue Cell Viability Reagent (Invitrogen) per the manufacturer's protocol. Metabolic activity was measured for resting splenic macrophages as well as 3 h following *Shigella* infection.

Caspase Cleavage Assays. ATG16L1 isoforms were cloned into pCDNA3.1(+)-Neo and in vitro translated in the presence of [^{35}S]methionine according to the manufacturer's instructions (Promega). ATG16L1 ^{35}S-labeled ATG16L1 proteins were incubated in the presence or absence of different human recombinant caspases in caspase cleavage buffer (20 mM Hepes, 100 mM NaCl, 10 mM DTT, pH 7.4) for 1 h at 37 °C. Reaction products were analyzed by SDS/PAGE followed by fluorography.

Autophagic Flux Analysis by Western Blotting. For Western blots, 1×10^5 MEFs were plated in 12-well format and treated for 1.5–2 h with complete IMDM or IMDM with 100 nM Torin 1 in the presence or absence of 10 μg/mL of E64d and pepstatin A (Sigma). For caspase inhibitor experiments, cells were treated overnight with DMSO, 20 μM caspase 3 inhibitor II (EMD Millipore), or 20 μM caspase 3/7 inhibitor I (EMD Millipore). Fresh DMSO or caspase inhibitors were then added at the time of stimulation. Cells were lysed in standard lysis buffer (25 mM Tris, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, protease inhibitors; Roche), and total protein content was quantified by using Pierce BCA assay. Normalized cell lysates were resolved by SDS/PAGE, and immunoblotting was performed by using mouse anti-actin (Sigma), rabbit anti-LC3b (Sigma), rabbit anti-beclin 1 (Santa Cruz), or rabbit anti-p62 (Cell Signaling). For Western blots in intestinal epithelial cells, colonic spheroids were isolated by using Cell Recovery Media (BD Biosciences) to remove Matrigel. For flux assays, organoids were treated with 100 nM bafilomycin A1 (Sigma) for 1 h. Spheroids were then pelleted and lysed in RIPA buffer with protease inhibitors (Sigma) and were sonicated. Samples were run on 15% (wt/vol) Tris·HCl gels (Bio-Rad) and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% (wt/vol) milk in 0.1% Tween 20 Tris-buffered saline solution for 1 h at room temperature and probed with rabbit anti-LC3 (Sigma) and mouse anti-GAPDH antibodies overnight at 4 °C. Blots were incubated for 1 h with HRP-conjugated secondary antibodies (Sigma) before development by using the SuperSignal West Dura chemiluminescent kit (Thermo Fisher Scientific).

Paneth Cell and Goblet Cell Assessments. The distal ileum and colon were removed, cut open along the length, and pinned on black wax. Tissue was fixed in 10% (wt/vol) formalin fixative overnight at 4 °C. Two-centimeter strips of intestinal tissues were embedded in agar to enrich for well-oriented crypt–villus units. Images were taken on an Olympus BX51 microscope. Immunohistochemistry for lysozyme was performed as previously described (7). Sections were viewed with a Zeiss Axiovert 200 inverted fluorescence microscope and quantified on an Olympus AX70 epifluorescence microscope. Periodic acid–Schiff (PAS)/Alcian blue staining was used to quantify goblet cells. Quantification of goblet cell size was performed by using ImageJ. Paneth cell phenotypes were classified as previously described (8). Cells were classified as normal if they contained numerous small (~1 μm) lysozyme-positive apically located granules. Cells were classified as disordered if they contained lysozyme-positive granules of normal size and quantity, but had some basally located granules. Cells were classified as diminished if they contained fewer than 10 granules, with the remaining granules frequently enlarged or fused. Cells were classified as diffuse if they did not contain any secretory granules and had diffuse lysozyme staining throughout the cytoplasm.

Interactome Analysis. We used the interactome-based affiliation scoring (IBAS) method to analyze the list of proteins found significant in the pull-down experiment (9). IBAS employs permutation and cross-validation to determine: (*i*) whether a set of phenotype-causing proteins (PCPs) involved in a phenotype are significantly more connected than by chance; and (*ii*) whether a candidate protein (Cp) significantly interacts with this set. If the interaction between a Cp and the PCPs is significant, a Cp is determined to be associated with the phenotype on the basis of its interactome; hence the name of the algorithm. Interactome data come from a well-established human protein interaction network (InWeb) of ~430,000 protein–protein interactions among 12,507 human proteins (6). The IBAS technique determined that this set predicted each other with an area under the receiver operating characteristic curve of 0.59 ($P < 0.01$ with a permutation test). Subsequently, all candidates (i.e., ~12,500 proteins covered by interaction data in InWeb, excluding the set of PCPs) are scored and ranked, and permutation tests were used to determine the significance of the observed scores. After extending the initial network with candidate proteins found to be significant, we carried out an enrichment analysis for specific modules based on hypergeometric testing.

Statistical Analysis. Comparisons between groups were performed by using the Student *t* test.

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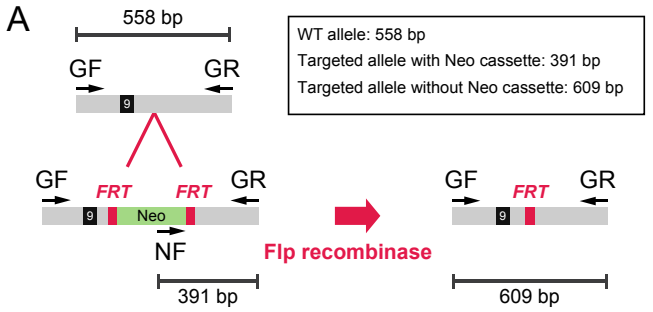
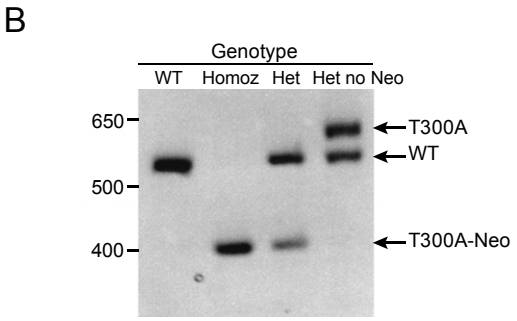
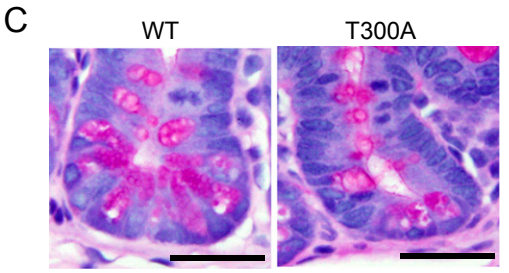
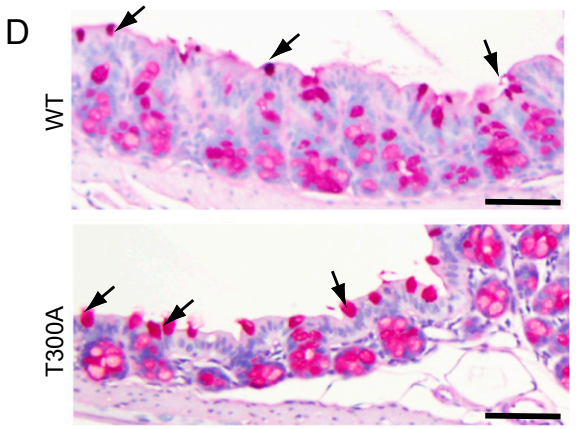
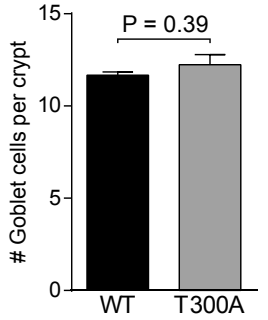


Mouse isoform	Accession number	Human isoform equivalent	Position of CD-associated polymorphism in mature mRNA
α or 3	NP_001192321.1	2	T281, exon 8
β or 2	NP_084122.2	1	T300, exon 9
γ or 1	NP_001192320.1	-	T316, exon 10









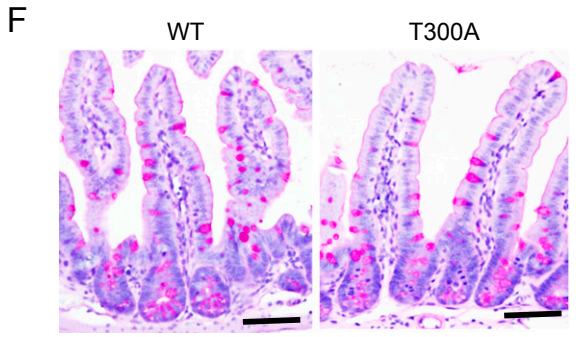


Fig. S1. Genotyping approach for construction of T300A knock-in mice. (*A*) Location of genotyping primers used in multiplexed PCRs and predicted sizes of PCR products indicating an unmodified allele (WT, 558 bp), targeted allele containing the neomycin cassette (T300A-Neo, 391 bp), or targeted allele without the neomycin cassette (T300A, 609 bp). Table shows murine isoforms of Atg16L1 and human equivalents. The β-isoform was used for numbering purposes. (*B*) Genomic DNA from mouse tails were analyzed by multiplexed PCR, yielding products of predicted size. (*C*) Higher magnification (60×) images of PAS-stained sections of small intestinal crypt base from WT and T300A mice to illustrate Paneth cell granule defects. (*D*) Higher-magnification image of ascending colon shown in Fig. 1*E* to illustrate increase in goblet cell size. (*E*) Quantification of the average number of goblet cells per crypt–cuff axis from ascending colon crypts in WT and T300A mice. No significant difference was found as determined by Student *t* test. (*F*) PAS-stained sections of small intestines from WT and T300A mice illustrating no differences in goblet cell size in the small intestine.

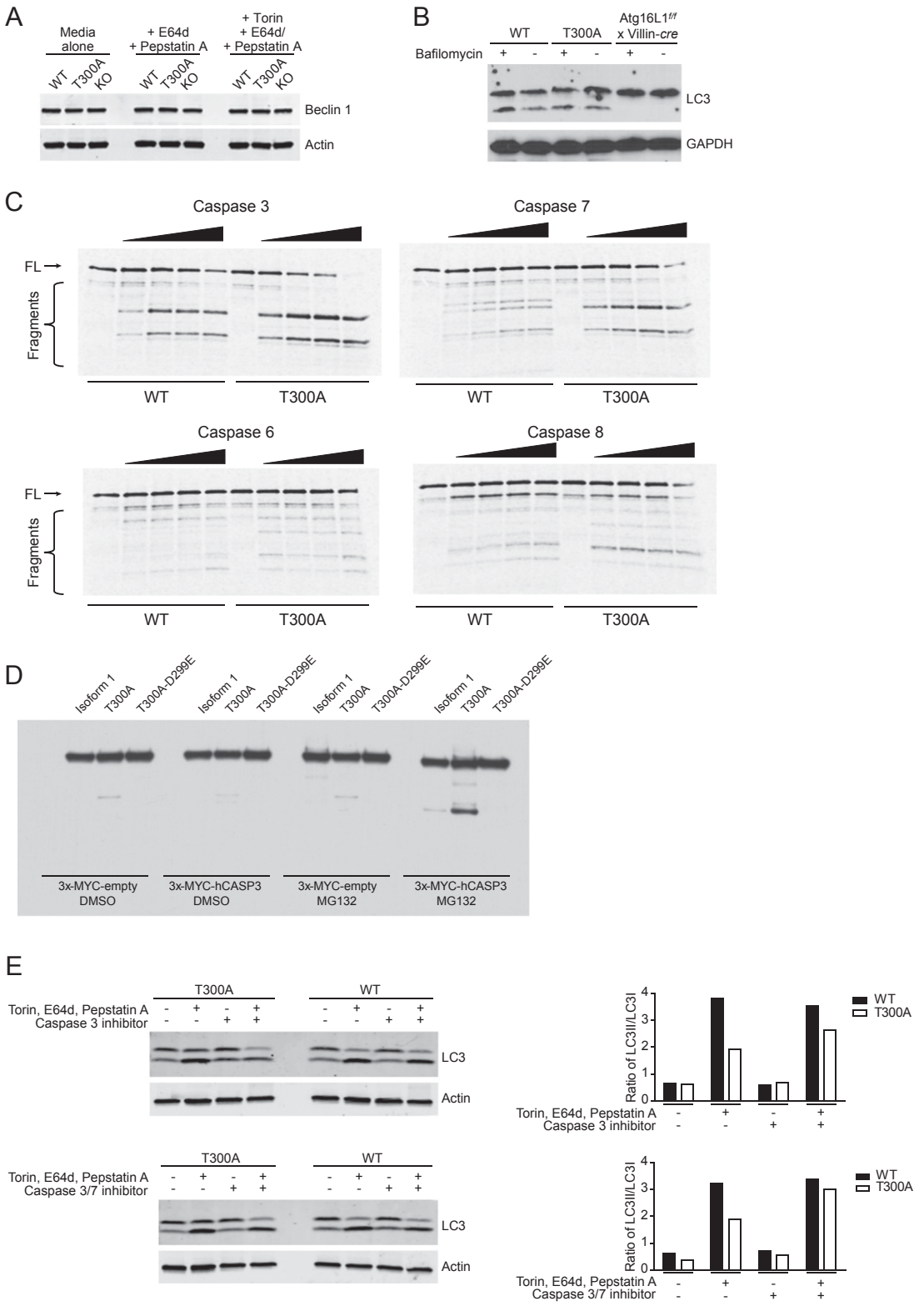
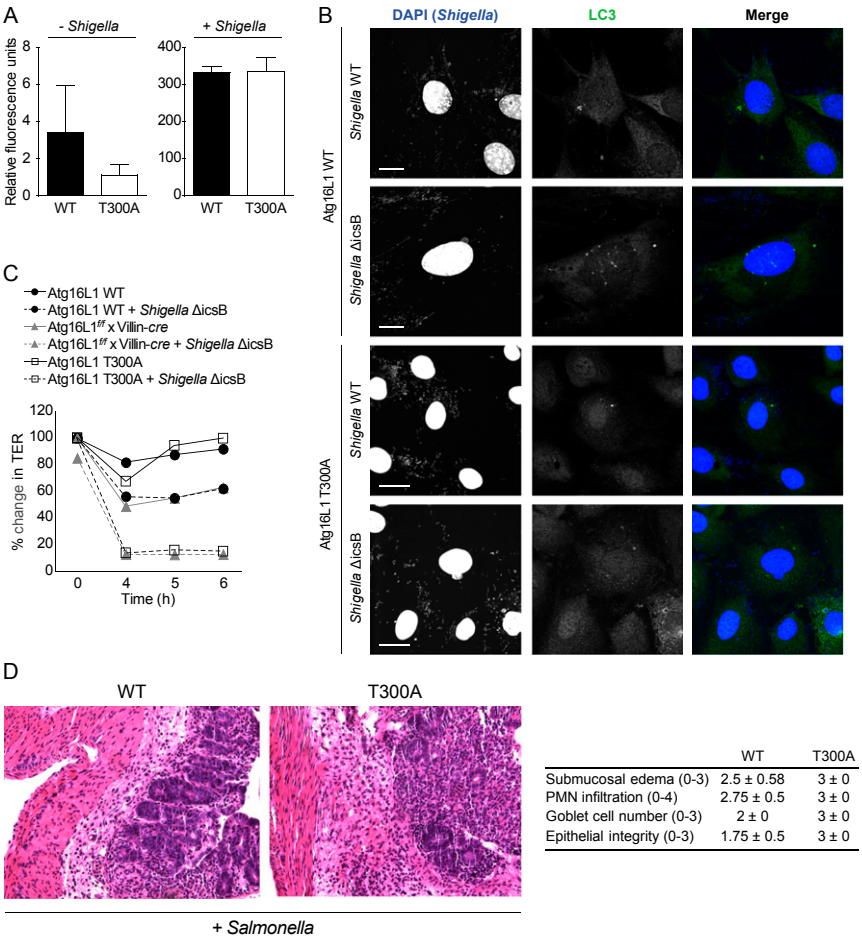


Fig. S2. ATG16L1 T300A results in decreased levels of LC3-II in primary cells and is more susceptible to cleavage by caspase 3 and caspase 7. (*A*) Immunoblot analysis of beclin 1 in MEFs untreated or stimulated for 4 h with 100 nM Torin 1, 10 μg/mL E64d, and 10 μg/mL pepstatin A. Blots were probed for beclin 1; actin served as a loading control. (*B*) Colonic cells were untreated or treated with 100 nM bafilomycin A1 and separated by SDS/PAGE. Blots were probed with anti-LC3 antibody; GAPDH served as loading control. (*C*) Caspase cleavage of overexpressed ATG16L1 in HeLa cells. Cells were cotransfected with increasing amounts of the indicated caspase constructs and a fixed amount of FLAG-tagged ATG16L1 WT or T300A. Western blot analysis was performed with anti-FLAG antibody. FL, full-length. (*D*) Human caspase 3 was overexpressed in Atg16L1$^{-/-}$ MEFs reconstituted with the indicated ATG16L1 expression vector. Cells were treated with DMSO or 25 μM MG132 for 6 h, and ATG16L1 cleavage was analyzed by Western blot using an anti-ATG16L1 antibody. (*E*) Immunoblot analysis of LC3-I/II in MEFs untreated or stimulated for 4 h with 100 nM Torin 1, 10 μg/mL E64d, and 10 μg/mL pepstatin A. Caspase 3 inhibitor II or caspase 3/7 inhibitor I were added as indicated at a final concentration of 20 μM. Blots were probed for LC3-I/II. Actin served as a loading control. Graph shows results from densitometry analysis using LI-COR software.



	WT	T300A
Submucosal edema (0-3)	2.5 ± 0.58	3 ± 0
PMN infiltration (0-4)	2.75 ± 0.5	3 ± 0
Goblet cell number (0-3)	2 ± 0	3 ± 0
Epithelial integrity (0-3)	1.75 ± 0.5	3 ± 0

Fig. S3. Responses to bacterial infection. (*A*) Metabolic activity of splenic macrophages before and 3 h after infection with *Shigella* using Alamar blue. WT and T300A splenic macrophages exhibited similar levels of metabolic activity, suggesting no differences in cell viability between the genotypes (data shown as mean ± SD; $n = 2$). (*B*) Colocalization of LC3 and *Shigella* in MEFs. Atg16L1 WT or Atg16L1 T300A MEFs were infected with WT *Shigella* or *Shigella* ΔicsB for 2 h. Cells were fixed and stained with anti-LC3 (green) or DAPI (*Shigella* and nuclei) to visualize colocalization of *Shigella* with the autophagy machinery. (Scale bars, 25 μm.) (*C*) Primary cultures of WT, T300A, or Atg16L1$^{f/f}$ × Villin-*cre* small intestinal epithelial cells were grown on transwells and infected with 1:100 dilution of ΔicsB *S. flexneri* at the apical and basolateral surfaces of the transwell. Transepithelial electrical resistance (TER) was monitored every hour. (*D*) Cecum was harvested 6 d after *Salmonella enterica* serovar Typhimurium infection for histological analysis. Representative H&E-stained sections are shown (20× magnification). Table breaks down histology score by category. The average score ± SD for each histological parameter is displayed.

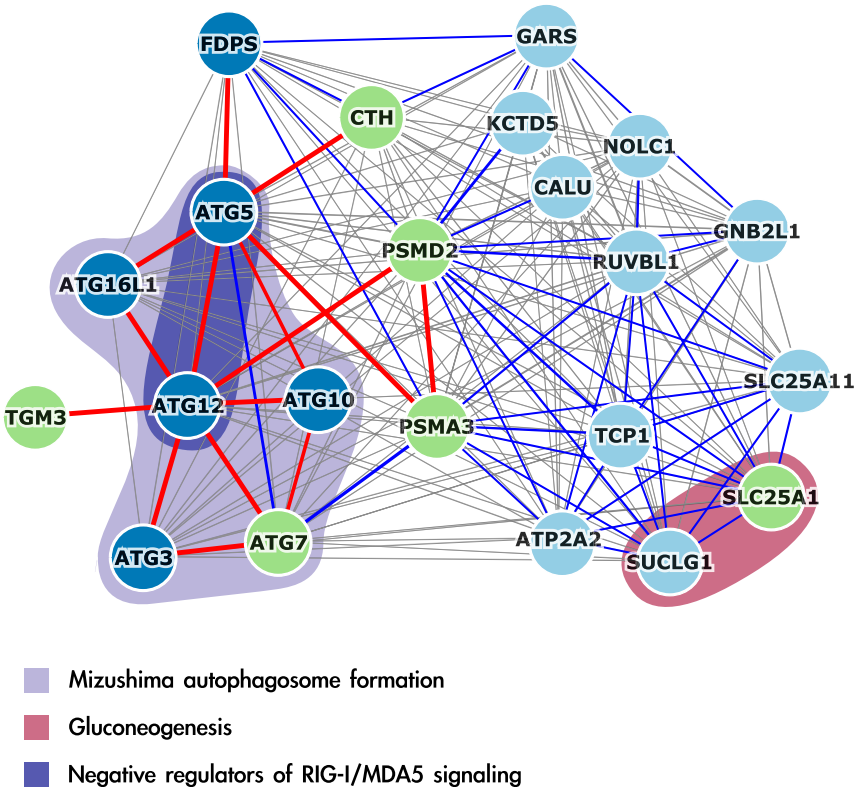


Fig. S4. Interactome analysis of proteomic hits. The image shows the network after extension and including indirect interactions (gray). High-confidence interactions are shown in red, and lower confidence interactions are visualized in blue. We highlighted three significant enriched modules found in this network, the Mizushima autophagosome formation ($P < 8.83 \times 10^{-7}$), gluconeogenesis ($P = 1.65 \times 10^{-6}$), and negative regulators of RIG-I/MDA5 signaling ($P = 1.37 \times 10^{-6}$).

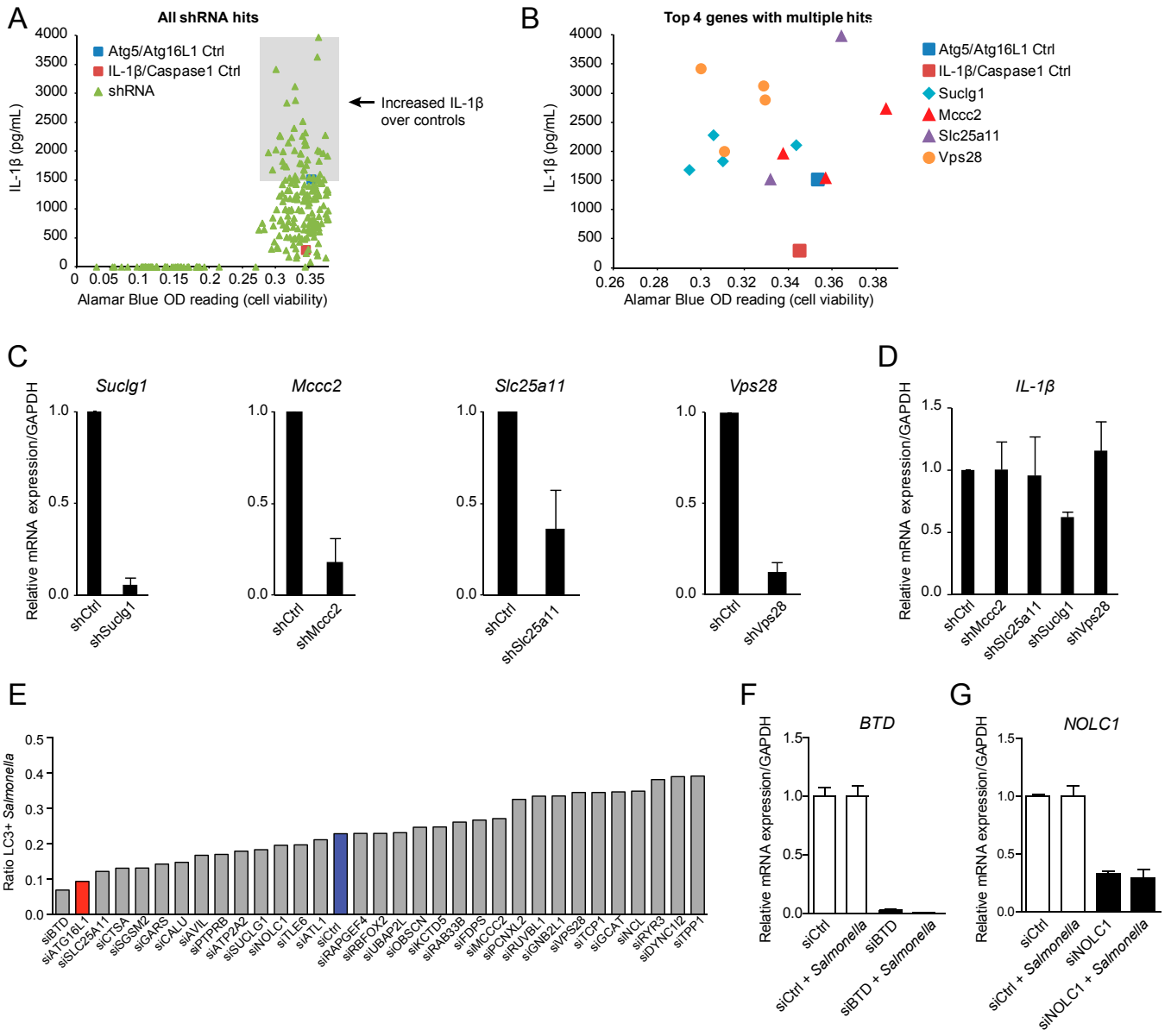


Fig. S5. Knockdown of ATG16L1 interactors in IL-1β secretion and antibacterial autophagy. (*A*) Summary plot of IL-1β secretion and Alamar blue staining in immortalized BMDMs transduced with shRNAs against ATG16L1 interactors. shRNAs against IL-1β and caspase 1 served as controls for negative regulators, and Atg5 and Atg16L1 served as controls for positive regulators. shRNAs that resulted in increased IL-1β production with effect size >100% of positive controls compared with negative regulators are shaded in gray. Data are representative of three independent experiments. (*B*) Summary of top scoring shRNAs as presented in *A*. (*C*) Quantitative real-time PCR analysis of BMDMs transduced as described in *A*. Relative mRNA levels calculated by $\Delta\Delta C_T$ method are shown (data shown as means ± SD; $n = 3$). (*D*) Relative Il-1β mRNA levels were assessed at steady state in BMDMs transduced as described in *A* (data shown as means ± SD; $n = 3$). (*E*) Summary of GFP-LC3-*Salmonella* colocalization in HeLa cells treated with the indicated siRNAs. Nontargeting siRNA was used as a negative control, and ATG16L1 served as a positive control. Data are representative of three independent experiments. (*F* and *G*) Relative mRNA levels of siRNA targets in cells treated with siRNA for 48 h at steady state or in the presence of *Salmonella* infection (data shown as means ± SD; $n = 3$).

Table S1. Primers used for Quantitative real-time PCR

Primer name	Primer sequence
BTD forward	CCTCACCGCTTCAATGACACA
BTD reverse	CCTTGGGTCACTGCTATGACAA
NOLC1 forward	TTCCTGCGCGATAACCAACTC
NOLC1 reverse	CCTGTAACTTTCGCTCTGGGA
NDP52 forward	ATTTCATCCCTCGTCGAAAGGA
NDP52 reverse	GGAAAGGAATACTTGCTCCCC
AVIL forward	ACAACGACCCTGGGATCATTG
AVIL reverse	GTCGAGAGGATGACGTAGCAG
VPS28 forward	GCCGGAGCTGTATGAGGAAG
VPS28 reverse	GGGGGTGACACAGTCCTTG
MCCC2 forward	GCCTATCACGGGGACTCAGT
MCCC2 reverse	CACTCCCTCCTAGTCTCACATAC
SLC25A11 forward	GTACCTCCCCTAAGTCTGTCAA
SLC25A11 reverse	CTGCATCCGGTTCTTCACCAG
SUCLG1 forward	TGGGATACGACACGGGTCTTA
SUCLG1 reverse	CAGAAGCCGTTGCTCCTGTT
CASPASE1 forward	ACAAGGCACGGGACCTATG
CASPASE1 reverse	TCCCAGTCAGTCCTGGAAATG
GAPDH forward	GGAGCCAAACGGGTCATCATCTC
GAPDH reverse	GAGGGGCCATCCACAGTCTTCT
IL-1B forward	GCCCATCCTCTGTGACTCAT
IL-1B reverse	AGGCCACAGGTATTTTGTCG
ATG5 reverse	GGAGGACACACTCTTTCAATCT
ATG5 forward	CAGGTGATGATTCACGGGATAG

Dataset S1. Results of proteomic iTRAQ analysis

Dataset S1