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Supplementary Information for

Cytosolic galectin-4 enchains bacteria, restricts their motility and promotes inflammasome activation in intestinal epithelial cells

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Other supplementary materials for this manuscript include the following:

Extended Materials and Methods

Cells

HT-29 cells (kindly gifted by Dr. Ching-Liang Chu of National Taiwan University, Taiwan) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1×NEAA, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. HeLa cells were cultured in DMEM containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% FBS at 37°C in 5% CO_2.To generate galectin-4-knockout HT-29 cells, the GeneArt CRISPR Nuclease Vector with an OFP Reporter Kit (Invitrogen A21174) was used according to the manufacturer's instructions. The target sequence of galectin-4 was designed using CHOPCHOP (https://chopchop.cbu.uib.no) (*SI Appendix*, Table S1). sgRNA was cloned into the GeneArt CRISPR Nuclease Vector. The resulting plasmid was transfected into HT-29 cells using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific). Single cells were sorted into a 96-well plate via fluorescence-activated cell sorting (FACS). Targeted cleavage and missense mutations were assayed by gene sequencing, and galectin-4 deficiency was confirmed by immunoblotting.

The pEGFP-C1 vector (Clontech 6084-1) and pEGFP-galectin-4 plasmids (the coding sequence of human galectin-4 subcloned into the pEGFP-C1 vector) were transfected into galectin-4-deficient HT-29 cells and HeLa cells using Lipofectamine™ 3000 Transfection Reagent. Reconstituted cells were sorted using a FACSAria III cell sorter (Becton, Dickinson and Company) and maintained in culture medium containing G418 (Gibco). The expression of EGFP and EGFP-galectin-4 in galectin-4-deficient HT-29 cells and in HeLa cells was verified by immunoblotting.

Bacteria

Escherichia coli O19ab (BCRC 15492), *E. coli* O86 (BCRC 15874), and *S. enterica* serovar Cubana (BCRC 15565) were purchased from the Bioresource Collection and Research Center, Taiwan. *S. enterica* serovar Typhimurium (SL1344) was a kind gift from Dr. Jr-Wen Shui of Academia Sinica, Taiwan. All bacterial strains were grown in lysogeny broth at 37°C with 200 RPM agitation, unless otherwise stated. To generate GFP-and RFP-expressing bacteria, a pLAC-GFP or pLAC-RFP plasmid (1) was introduced into *S. enterica* serovar Worthington by electroporation (BTX ECM 630 Exponential Decay Wave Electroporation System), and bacteria were cultured in lysogeny broth with 30 μg/mL chloramphenicol (Sigma).

Recombinant human galectin-4 (rhGal4) and recombinant human galectin-9 without a linker region (rhGal9null)

The coding sequence of full-length (1–323 amino acids) human galectin-4 was cloned into the pET-28b vector (Novagen). After the plasmid was transformed into ClearColi® BL21(DE3) electrocompetent cells (Lucigen), several colonies were isolated and grown in 2x YT medium. Protein synthesis was induced by the addition of 1 mmol/L isopropyl-β-d-thiogalacto-pyranoside (Bio Basic, Inc.). The cell pellets were suspended in TES buffer (20 mM Tris HCl, 5 mM EDTA, and 10 mM sucrose, pH 7.5) and disrupted by sonication

(Qsonica Sonicator Q700). Recombinant proteins were purified by affinity chromatography on lactosyl sepharose, and bound proteins were eluted with 250 mM lactose in PBS. The eluted fractions were collected and dialyzed against PBS. Recombinant human galectin-9 without a linker region, rhGal9null (2), was provided by the Glycobiology Core at the Institute of Biological Chemistry in Academia Sinica. Fluorescent-labeled human galectin was prepared using the Lightning-Link Rapid Atto 488 Labeling kit (Innova Biosciences) or Lightning-Link Texas Red Labeling kit (Innova Biosciences).

Mice

Heterozygous and homozygous human galectin-4 transgenic (hGal4-Tg) mice were identified by quantifying the amount of transgene relative to that of GAPDH from genomic DNA using quantitative PCR. The mice were maintained under specific pathogen-free conditions at the Institute of Biomedical Sciences, Academia Sinica, Taiwan. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Academia Sinica, Taiwan (IACUC Protocol number: 17-09-1111).

Real-time quantitative PCR

Total RNA was extracted from the ileum and colon of wild-type mice using the RNeasy kit (Qiagen, Hilden, Germany). mRNA was then reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA), and the levels of mouse *galectin-1, 3, 4, 7, 8, 9* and *gapdh* were determined by quantitative real-time PCR using specific primers (*SI Appendix* Table S1) and Universal Probe Library probes (Roche Holdings AG, Basel, Switzerland) targeting the gene products according to the manufacturer's instructions. Relative galectin mRNA levels were calculated using the delta cycle threshold method.

CFU assay

HT-29 cells were seeded overnight in 24-well plates at a density of 5×10^5 cells/well. 500 nM wortmannin (Sigma-Aldrich) was added to the cell culture medium 1 h before infection and was present throughout the experiment. *S. enterica* was grown overnight at 37°C in LB and then the cultures were diluted 1:20 in fresh LB without antibiotics and incubated for 3 h at 37°C with shaking at 200 RPM. *S. enterica* in OPTI-MEM (Gibco) was added to the cell culture, followed by centrifugation at 300 x g for 5 min. Cells were infected with bacteria for 1 h at 37°C. Extracellular bacteria were killed by treating the cells with 100 μg/mL gentamicin (Sigma-Aldrich) for 30 min, followed by 10 μg/mL gentamicin throughout the rest of the experiments. Cells were lysed at 2 h and 10 h post-infection, the number of intracellular bacteria was determined by plating serial dilutions onto LB agar plates. To quantify the number of cytosolic bacteria in HT-29 cells, a chloroquine protection assay was used as previously described (3). Chloroquine (Sigma-Aldrich, 400 μM) was added 1 h before cells were lysed and the number of viable bacteria in the cytosol was determined by plating serial dilutions onto LB agar plates.

Bacteria were incubated in LB containing 100 μg/mL bovine serum albumin (BSA) or rhGal4 at 37°C for 1 h with 250 RPM or without agitation. Under static condition, bacteria were incubated in LB containing 50 μg/mL BSA, rhGal4, or rhGal9null at 37°C for 2 h. For

determining the number of viable bacteria, bacterial suspension was serially diluted with PBS containing 20 mM lactose in order to prevent bacterial aggregation caused by galectin-4 and -9, and then placed on LB agar plates.

Enzyme-linked Immunosorbent Assay (ELISA)

The luminal contents of the stomach and different parts of the small intestine, cecum, and colon were collected from sacrificed mice by flushing with 2 mL of sterile PBS containing protease inhibitors. The lavage fluids were centrifuged at 14,500 x g for 5 min at 4°C to remove the particulate components of luminal contents. The amount of galectin in the flushed solutions was determined using ELISA. The specific paired antibodies used to detect galectin-1, -3, -4, -7, and -9 were purchased from R&D Systems (galectin-1: AF1245 and BAF1245, galectin-3: MAB1197 and BAF1197, galectin-4: AF2128 and BAF2128, galectin-7: MAB1308 and BAF1304, galectin-9: AF3535, and BAF3535). Mouse galectin-8 levels were determined using an ELISA kit (Cloud-Clone Corp.).

Feces were weighed and homogenized in 200 µL sterile PBS with proteinase inhibitors by bead-beating (3 mm steel ball; 25 Hz for 1 min in a TissueLyser [Qiagen]). The homogeneous mixtures were centrifugated at 14,500 x g for 5 min at 4°C to remove the particulate component. The amount of human galectin-4 was determined by ELISA, which was performed using 1 µg/mL of anti-human galectin 4 (GeneTex 90264, customized) as the capture antibody, and 1 µg/mL of biotinylated anti-human galectin-4 (R&D BAF1227) as the detection antibody.

Microscopy imaging and Immunofluorescence assays

GFP- and RFP-expressing bacteria were incubated in LB media containing 50 µg/mL rhGal4 or rhGal9null at 37°C for 2 h. The bacterial suspension was then placed on a slide, covered with a cover slide, and observed under an LSM 700 laser scanning confocal microscope (ZEISS). To observe bacteria without fluorescence, images were acquired using an Olympus BX43 microscope (Olympus).

Normal mouse intestinal tissues were fixed in 4% paraformaldehyde overnight and embedded in optimum cutting temperature medium (Tissue-Tek) for immunofluorescent staining. 10 µm cryosections on the slide were fixed in acetone at −20°C for 10 min and washed in PBS. Fluorescence in situ hybridization for commensal bacteria was performed at 46°C for 6 h in 0.9 M NaCl, 100 mM Tris-HCl, pH 7.4, 0.01% SDS with 5 ng/mL FITC-labeled universal bacterial probe (GCTGCCTCCCGTAGGAGT). The slides were washed at 46°C in wash buffer (0.9 M NaCl, 100 mM Tris-HCl, pH 7.4) and then in PBS. Next, the slides were incubated with goat anti-mouse galectin-4 (R&D AF2128) at 4°C overnight. The slides were washed in PBS, incubated with Alexa 555 donkey anti-goat IgG (Invitrogen), and mounted using water-soluble DAPI Fluoromount-G (Southern Biotech). To stain the cryosections of the infected hGal4-Tg ileum tissue, the slides were incubated with goat anti-human galectin-4 (R&D AF1227) and rabbit anti-*Salmonella* O antigens (Difco 222611) at 4°C overnight. The slides were washed in PBS, incubated with Alexa 488 donkey anti-goat IgG (Invitrogen) and Alexa 555 donkey anti-rabbit IgG (Invitrogen), and mounted using water-soluble DAPI Fluoromount-G (Southern Biotech). Images were obtained using an LSM 700 or LSM 880 laser scanning confocal microscope (ZEISS).

HT-29 and HT-29-Gal4KO cells were cultured on 12-mm glass slides overnight in a 24-well culture dish. Cells were washed twice with PBS and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. 0.5% Triton X-100 in PBS was used for permeabilization for 10 min, and cells were blocked with 3% bovine serum albumin for 30 min to prevent nonspecific binding of antibodies. Cells immobilized on glass slides were incubated with goat anti-human galectin-4 (R&D AF1227), rabbit anti-*Salmonella* O antigens (Difco 222611), and mouse anti-phospho-NLRC4 (Ser533) (Thermo Fisher Scientific MA5-31846) overnight at 4°C. Cells were washed three times with PBS and stained with corresponding fluorescent-labeled secondary antibody in appropriate antibody diluents for 1 h at room temperature. Slides were mounted for fluorescent nuclear staining using water-soluble DAPI Fluoromount-G (SouthernBiotech). The stained slides were analyzed using an LSM 880 Laser Scanning Confocal Microscope (ZEISS), and image processing was performed using the ZEN Image Analysis module.

Live-bacteria imaging

To monitor live bacteria cultured with recombinant human galectin-4 in vitro, bacteria were grown overnight at 37°C in LB. The overnight cultures were then diluted 1:20 in fresh LB without antibiotics and incubated for 3 h at 37°C with shaking at 200 RPM. Bacteria were cultured with BSA, rhGal4 or rhGal9null in LB for 10 min in 35 mm glass culture dishes. Time-lapse live-cell movies and images were captured with Axiovert 200M (ZEISS) using a Plan Apochromat ×100 (1.4 NA) objective in a 37°C incubator chamber. Images were processed using the ZEN image analysis module. Images were acquired at 100 fps for the measurement of bacterial motility. The instantaneous velocity of each motion of the bacterium was measured, and the resultant tracking data were analyzed using the Metamorph software (Molecular Devices).

Immunoblotting

Cells were lysed in lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1% Triton X-100, 150 mM NaCl) containing protease inhibitor cocktail. The lysates were centrifuged at 14,000 RPM for 10 min at 4°C, and the supernatants were collected. To recover protein from cell culture supernatants, the chloroform-methanol protein precipitation method was used as described (4). Cell lysate and precipitated protein from cell culture supernatant were then separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories). Membranes were blocked with 5% milk in PBS-0.5%Tween 20 (PBS-T) for 1 h, and then a 1:1000 dilution of mouse anti-caspase-1 mAb (14F468) (Santa Cruz; sc-56036), 1:1000 dilution of biotin-conjugated anti-IL-18 monoclonal antibody (Invitrogen; BMS267-2MST) for activated IL-18, 1:1000 dilution of rabbit anti-IL-18 pAb (MBL; PM014) for pro-IL18, 1:1,000 dilution of goat anti-EGFP antibody (Abcam ab111258), 1:1000 dilution of goat anti-human galectin-4 Ab (R&D; AF1227), 1:5000 dilution of mouse anti-β-actin (Proteintech; 60008-1-Ig), or 1:5000 dilution of mouse anti-GADPH (Proteintech; 60004-1-Ig) with 3% BSA in PBS-T was used to probe the membrane at 4°C overnight. The membranes were washed with PBS-T to remove unbound primary antibodies and incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The

membranes were washed, developed with Amersham ECL prime luminal enhancer solution (Cytiva, RPN2232) or Luminata crescendo western HRP substrate (Millipore), and visualized using the Automated BioSpectrum Imaging System (Ultra-Violet Products). When the same membrane was stripped and reprobed with a different antibody, it was described in the respective figure legends. ImageJ was used to perform densitometric analysis to quantify the ratio of activated IL-18 to pro-IL-18 and p10 caspase-1 to caspase-1 in the blots.

Flow cytometry

Bacteria were grown overnight at 37°C in LB, and then the overnight cultures were diluted 1:20 in fresh LB without antibiotics and incubated for 3 h at 37°C with shaking at 200 RPM. Bacteria were washed, resuspended in PBS, and incubated with 10 μg/mL fluorescent-labeled rhGal4 or rhGal9null at 4°C for 1 h. Then, the bacteria were washed with PBS, 25 mM thiodigalactoside (TDG), or sucrose. Bacteria were incubated with 10 μg/mL of mouse galectin-4 (R&D 2128-GA) at 4°C for 1 h and then washed with PBS, 25 mM TDG, or sucrose. After the washing step, bacteria were stained with goat anti-mouse galectin-4 antibody (R&D AF2128) and Alexa 555 donkey anti-goat antibody. After washing again, bacteria were fixed in 4% paraformaldehyde and analyzed using a flow cytometer (BD LSRII). Data were analyzed using FlowJo software (BD Biosciences).

Two-photon microscopy

The mice were anesthetized with isoflurane and placed on a stage heated to 37°C. The small intestines of live mice were removed from the open abdominal cavity. GFP-expressing *S. enterica* serovar Worthington was injected directly into the ileum of wild-type and hGal4-Tg mice. The intestines were gently massaged to mix the injected solution with intestinal content. After equilibration for 30 min, luminal GFP-expressing *S. enterica* serovar Worthington was tracked individually in the intact intestine using a FVMPE-RS multiphoton laser scanning microscope (Olympus) equipped with a Chameleon Vision II laser (Coherent). An excitation wavelength of 980 nm and green filter (495–540 nm) were used to detect the GFP signal. The 25x objective (XLPLN25xWMP2) was used, and fluorescence images were acquired at 15 fps. The instantaneous velocity of each motion of the bacterium was analyzed using Imaris 9.3.

FITC-dextran permeability

IEC permeability was measured as previously described (5). Control wild-type and hGal4-Tg/Tg littermates were obtained from hGal4-Tg heterozygous breeders. Male and female mice aged 6–12 weeks were used in the experiments. The mice were administered 2.5% dextran sodium sulfate (DSS, MP Biologicals, molecular mass 36–40 kDa) in drinking water for 5 days. Seven-days after the start of DSS administration, the mice were fasted for 3 h and then orally gavaged with 150 μL of 80 mg/mL 4kDa FITC–dextran (Sigma FD4) in sterile water. Unused FITC-dextran was serially diluted with water to obtain a standard curve. Four-hours later, peripheral blood was collected from the mice and centrifuged at 10,000 × g at room temperature for 10 min. The serum and standard curve samples were added to 96-well plates, and the fluorescence intensity was measured with

excitation at 485 nm and emission at 528 nm. The permeability values were calculated based on a standard curve and normalized to mouse weight.

High-content imaging analysis

HeLa-EGFP-Gal4 and HeLa-EGFP cells were plated on CellCarrier-96 plates (PerkinElmer) at a density of 5 × 10^4 cells/well in 100 µL culture medium overnight. Cells were infected with *Salmonella* for 1 h at 37°C. Extracellular bacteria were killed by treating the cells with 100 µg/mL gentamicin (Sigma-Aldrich) for 30 min, followed by 10 µg/mL gentamicin throughout the rest of the experiments. For quantification of the proportion of cells containing galectin-4-coated bacteria, after infection for 3 h, images were taken at 40X magnification every 30 min for 16 hours and analyzed with ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices) equipped with an environmental control chamber (37°C, 5% CO_2). The total number of viable cells and cells that contained galectin-4-coated bacteria were determined using MetaXpress Software Cell Scoring Application Module. To calculate the percentages, the number of cells containing galectin-4-coated cytosolic bacteria was divided by the total cell count.

Lipopolysaccharide (LPS) extraction from *S. enterica* serovar Worthington and Bio-Layer Interferometry (BLI) assay

LPS was purified by the conventional phenol-water extraction method as previously described (6). Briefly, bacterial cells were washed and suspended in a final concentration of 45% phenol-water solution. The water phase containing LPS was lyophilized. LPS was isolated, then treated with RNase, DNase and proteinase K for further purification. After the enzymatic reaction, enzymes were removed by heating at 100°C followed by centrifugation with 10000 x g for 15 min.

Binding affinity between rhGal4 and LPS was measured using the Octet Red System (FortéBio, Fremont, CA, USA). Biotinylated rhGal4 was prepared by adding a 1:1 molar ratio Sulfo-NHS-LC-LC-Biotin (Thermo Fisher Scientific) to protein for 30 min at 4 °C, and the unreacted biotin was removed by using a 7K MWCO spin column (Thermo Fisher Scientific). Super Streptavidin (SSA) Biosensors (FortéBio) from FortéBio was hydrated in BLI rehydration buffer for 10 min in a 96-well plate, followed by loading with 1 µM biotinylated galectin-4 at 1000 rpm for 1200 s at 25 °C. For blocking unoccupied binding sites on SSA, the SSA biosensors were treated with biocytin (1 µg/mL) and then washed with PBS. The SSA biosensors were dipped in the assay buffer containing extracted *S. enterica* serovar Worthington LPS. The measurements were carried out using 600 s association and 600 s dissociation steps. All data were processed using Octet data analysis software, version 7.0 (FortéBio).

Immunohistochemistry

Formalin-fixed, paraffin-embedded intestinal tissue sections of wide-type mice and hGal4-Tg mice were dewaxed, rehydrated, then subjected to antigen unmasking in Epredia™ Lab Vision™ Citrate Buffer (Thermo Fisher Scientific). Endogenous peroxidase was quenched by UltraVision Hydrogen Peroxide Block (Thermo Fisher Scientific) for 15 minutes. Histofine Mousestain Kit Blocking reagent A (NICHIREI Bioscience) was applied

for 1 hour to block tissue endogenous mouse IgG. Human galectin-4 was detected by incubation with a mouse anti–human galectin-4 monoclonal antibody (R&D MAB1227) at 4°C overnight. Blocking endogenous mouse IgG by Histofine Mousestain Kit Blocking reagent B (NICHIREI Bioscience) for 10 minutes, the histological slides were then incubated in Simple Stain Mouse MAX PO (M) (NICHIREI Bioscience) for 30 minutes. The staining reaction for human galectin-4 was visualized by means of the ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories), and positive signals of DAB chromogen were developed as red-brown precipitates. The slides were counterstained with hematoxylin to detect the nuclei. Slied dewaxing, rehydration, and mounting were assisted by the Pathology Core Laboratory (IBMS, Academia Sinica). Human normal tissue array slides purchased from SUPER BIO CHIPS (Cat. No. AC, normal organs from non-cancer patients) were first dried for 1 hour at 60°C, then dewaxed, rehydrated, and subjected to antigen unmasking in Epredia™ Lab Vision™ Citrate Buffer (Thermo Fisher Scientific). Endogenous peroxidase was quenched by UltraVision Hydrogen Peroxide Block (Thermo Fisher Scientific) for 15 minutes. Human galectin-4 was detected by incubation with a mouse anti–human galectin-4 monoclonal antibody (R& D MAB1227) at 4°C overnight. After 0.1% PBST washing, the tissue array slides were incubated in Simple Stain Mouse MAX PO (M) (Nichirei Bioscience) for 30 minutes. The staining reaction for human galectin-4 was visualized by means of the ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories), and positive signals of DAB chromogen were developed as red-brown precipitates. The slides were counterstained with hematoxylin to detect nuclei. Slied dewaxing, rehydration, and mounting were assisted by Pathology Core Laboratory at Institute of Biomedical Sciences, Academia Sinica.

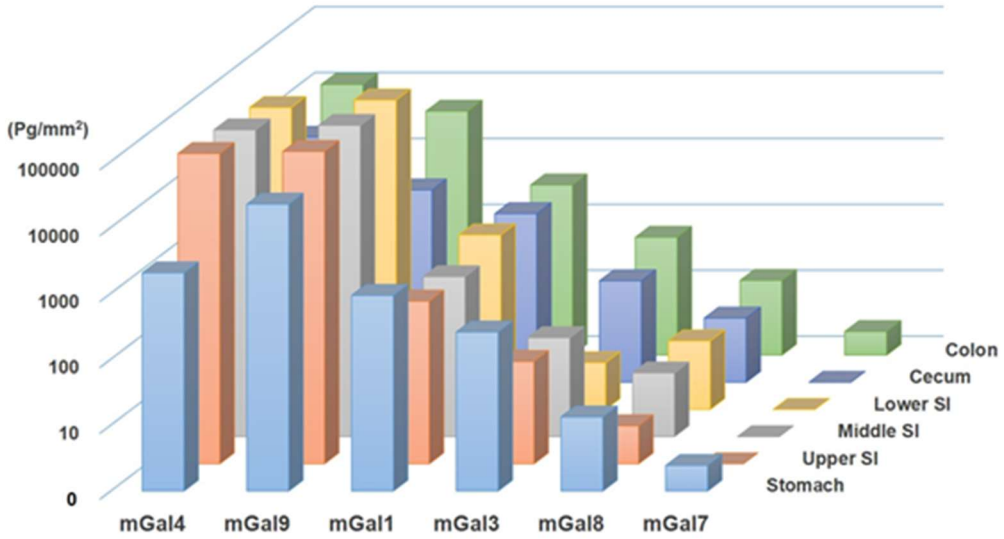


Fig. S1. Galectins are present in secretions from freshly isolated gastrointestinal tracts from mice. The stomach and different parts of the small intestine, cecum, and colon were isolated from wild-type C57BL/6 mice. The lumens were flushed with PBS containing protease inhibitors. The expression levels of different galectins were quantified in secretions via ELISA. The quantities of galectins were normalized to the surface area of the tissues. mGal, mouse galectin; SI, small intestine. Data represent the mean of three mice.

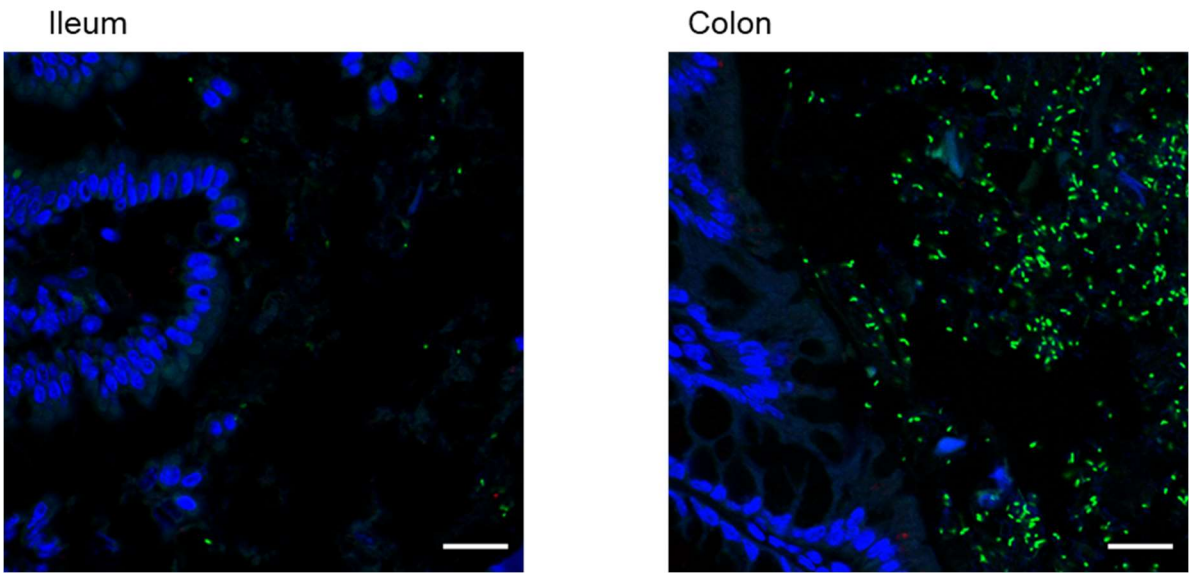


Fig. S2. The images of isotype control staining conducted in Fig. 1B. Immunofluorescence and fluorescence in situ hybridization staining of ileum and colon sections from a wild-type mouse. DAPI (blue), commensal bacteria detected by the universal bacterial probe (green), and isotype antibody corresponded to anti-mouse galectin-4 antibody. Scale bars, 20 μm.

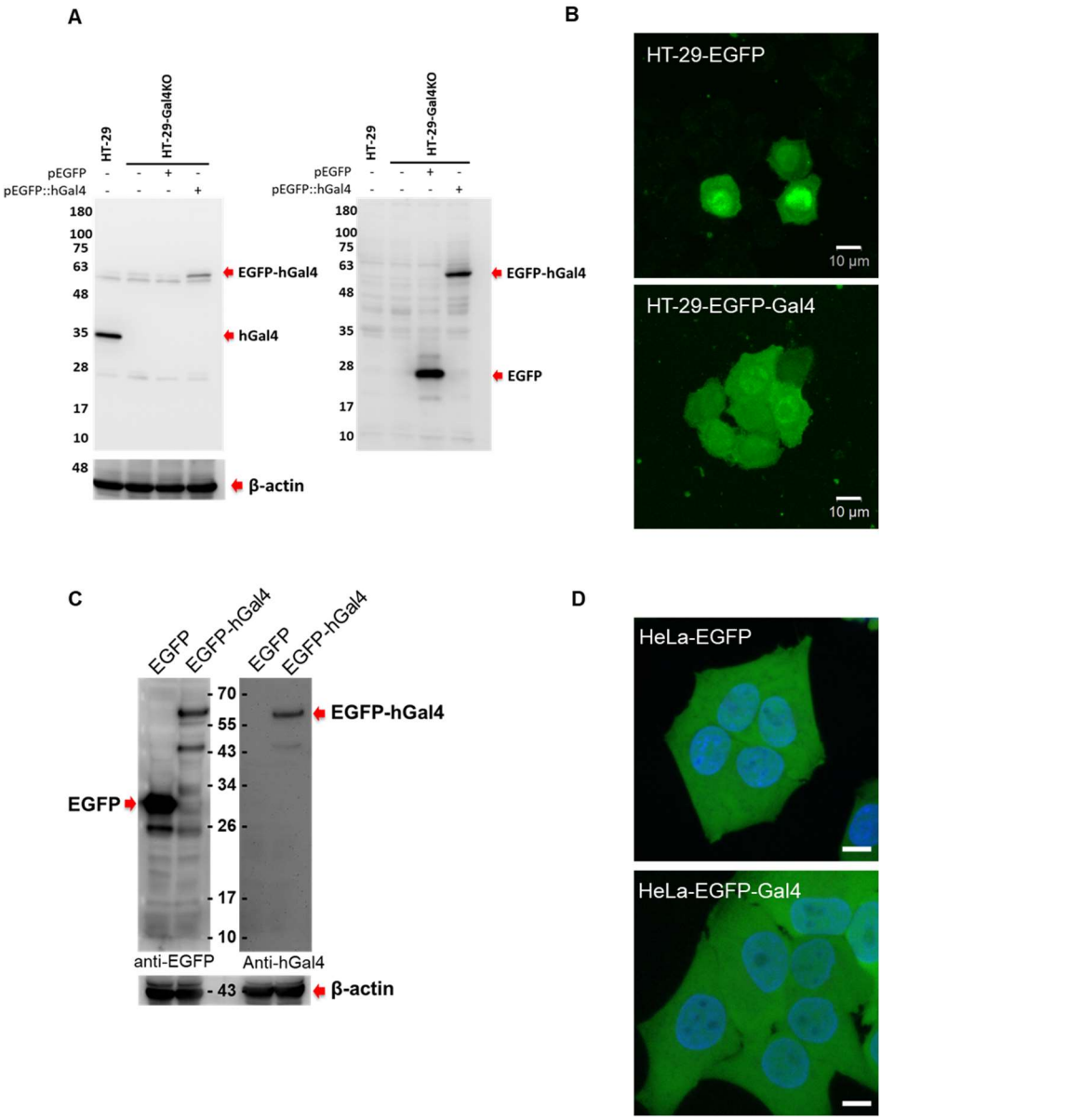
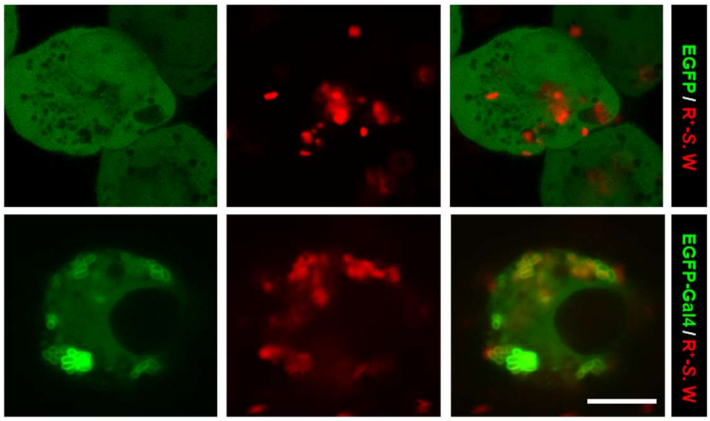


Fig. S3. (A) CRISPR knockout of the galectin-4 gene in HT-29 cells, and reconstitution of EGFP-Gal4 or EGFP into galectin-4-knockout HT-29 cells. Cell lysates were analyzed by immunoblotting with anti-human galectin-4 antibody, anti-β-actin; and the membrane was stripped and reprobed with anti-EGFP antibody. (B) The basal expression of EGFP-galectin-4 and EGFP in uninfected HT-29 cells. (C) Reconstitution of EGFP or EGFP-Gal4 into HeLa cells. Cell lysates were analyzed by immunoblotting with anti-human galectin-4, anti-EGFP and anti-β-actin antibodies. (D) The basal expression of EGFP-galectin-4 and EGFP in uninfected HeLa cells. Hoechst (blue). Scale bars, 10 μm.

A



B

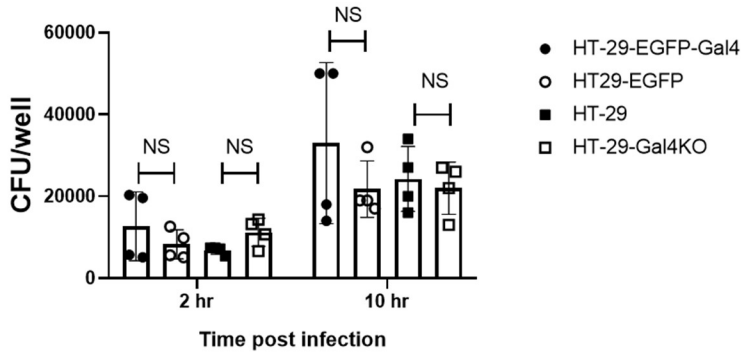


Fig. S4. (A) Intracellular galectin-4 coats cytosolic bacteria in HT-29-EGFP-Gal4 cells without wortmannin treatment.Cells were infected with RFP-expressing *S. enterica* serovar Worthington (R^+-*S.* W), MOI = 100. Images were acquired 10–12 h post-infection as shown. Scale bar, 10 μm. (B) Cells treated with wortmannin were infected with *S. enterica* serovar Worthington, MOI = 100. After 2 h and 10 h of infection, the intracellular bacteria were quantified by CFU enumeration. Data represent the mean ± SD. the *P*-value was calculated using the Mann-Whitney test; NS, not significant. Data are representative of two independent experiments.

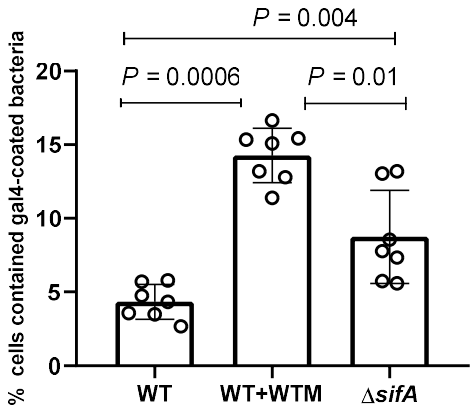


Fig. S5. The proportion of cells containing galectin-4-coated bacteria in HeLa cells. HeLa-EGFP-Gal4 cells treated with or without wortmannin were infected with WT or *sifA* mutant (Δ*sifA*) of *S. enterica* serovar Worthington, MOI = 100. From 3–16 h of infection, the total number of viable cells and cells containing galectin-4-coated bacteria were determined using MetaXpress Software Cell Scoring Application Module. Data represent the mean ± SD. the *P*-value was calculated using the Mann-Whitney test. Data are representative of two or three independent experiments.

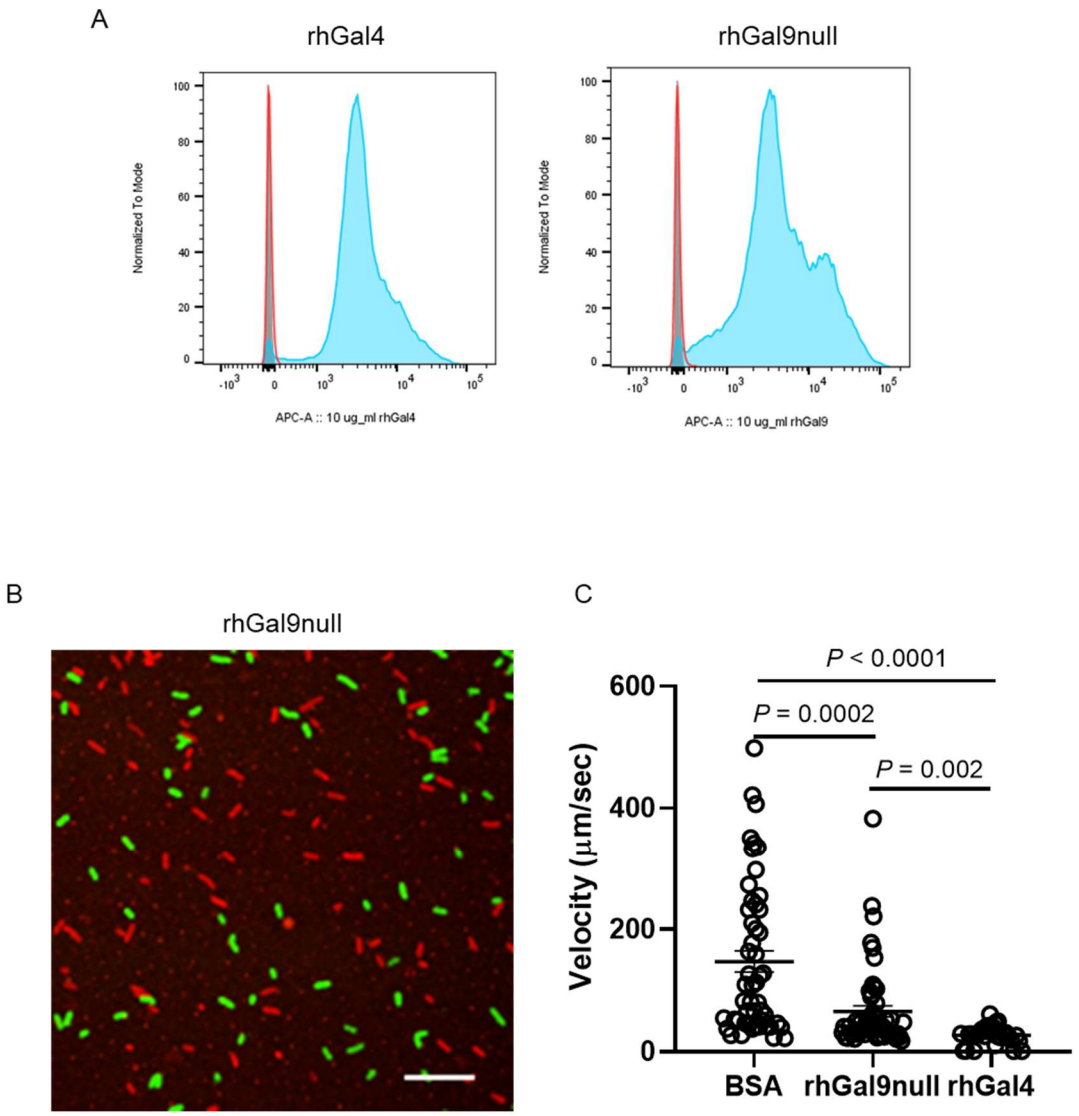


Fig. S6. (A) *S. enterica* serovar Worthington were incubated with 10 μg/mL APC-labeled rhGal4 or rhGal9null at 4°C for 1 h. The bacteria were washed with 25 mM of thiodigalactoside (red line), or sucrose (blue line). Galectin binding was determined by flow cytometry. (B) GFP- and RFP-expressing bacteria were incubated in LB media containing 50 μg/mL rhGal9null at 37°C for 2 h. Then the bacterial suspension was observed under microscope. Scale bars, 20 μm. (C) *S. enterica* serovar Worthington were incubated in LB containing 50 μg/mL BSA, rhGal4 or rhGal9null at room temperature for 10 min. Bacterial motility was tracked and calculated. Data represent the mean ± SEM, and the *P*-value was calculated using the Mann-Whitney test. Data are representative of two or three independent experiments.

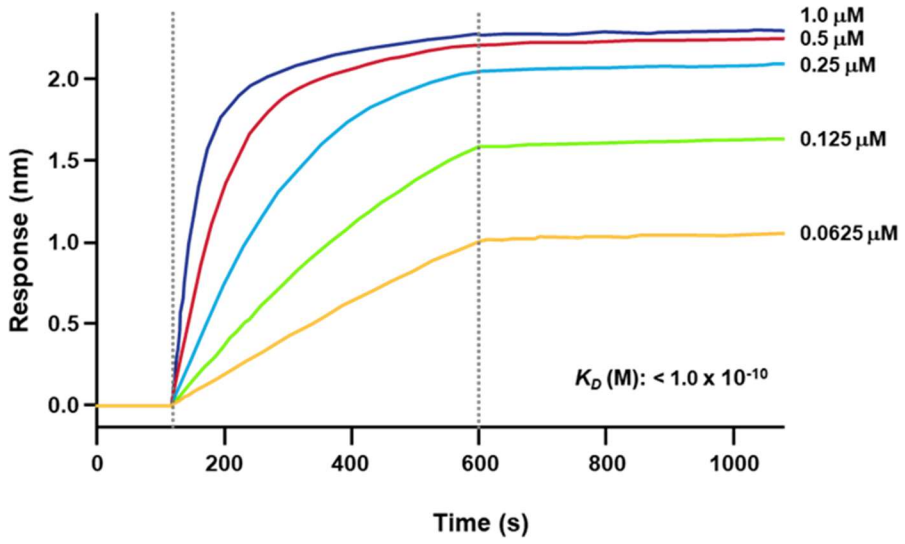


Fig. S7. Immobilized galectin-4 interaction with LPS of *S. enterica* serovar Worthington indicated by BLI approach. The binding capacity of galectin-4 to LPS by BLI measurements as indicated. Recombinant galectin-4 was immobilized on the SSA biosensors. The LPS concentrations were 0.0625, 0.125, 0.25, 0.5, and 1.0 µM (from bottom to top) for galectin-4. The curves are representative of at least two independent experiments and the calculated dissociation constants K_D were shown.

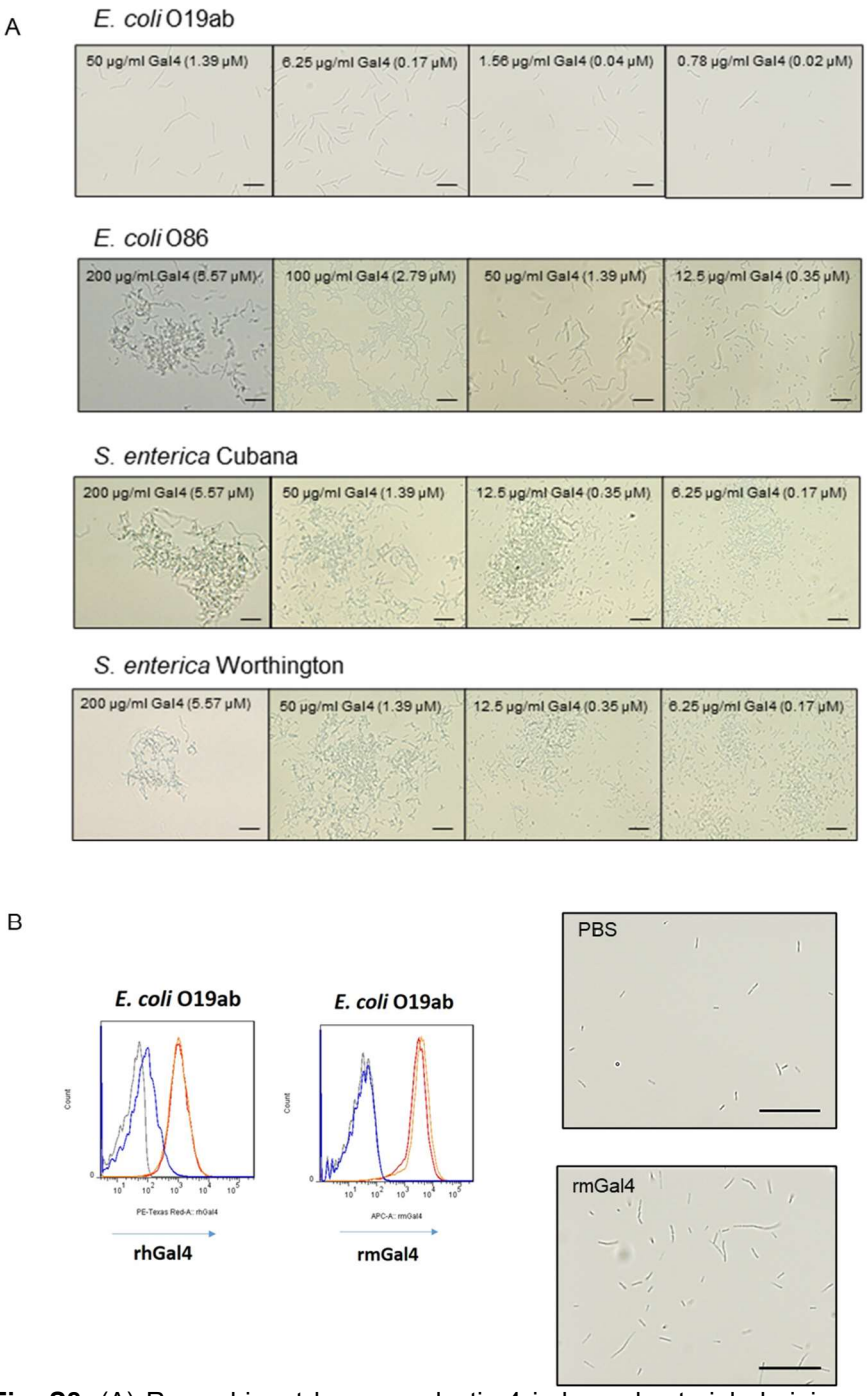


Fig. S8. (A) Recombinant human galectin-4 induces bacterial chaining and clustering. Bacteria were cultured in LB containing different concentrations of galectin-4 at 37°C for 3 h. Scale bars, 10 μm. (B) *E. coli* O19ab were incubated with 10 μg/mL Texas-Red-labeled recombinant human galectin-4 (rhGal4) or 10 μg/mL of recombinant mouse galectin-4 (rmGal4) at 4°C for 1 h. The bacteria were washed with phosphate-buffered saline (PBS) (red line), 25 mM of thiodigalactoside (blue line), or sucrose (orange line). Anti-mouse galectin-4 antibody was used to detect the binding of mouse galectin-4. Enchained *E. coli* O19ab were observed when cultured with 50 μg/mL of rmGal4 at 37°C for 3 h. Scale bars, 20 μm.

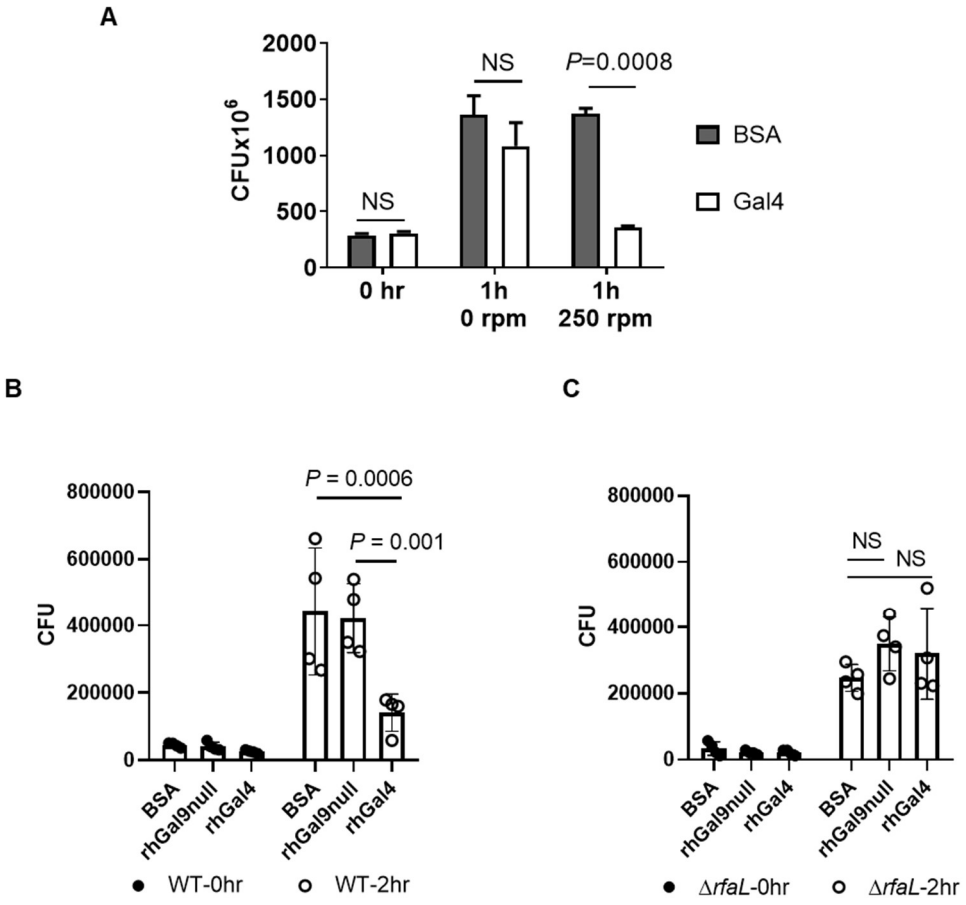


Fig. S9. (A) Bacteria were incubated in LB containing 100 µg/mL bovine serum albumin (BSA) or recombinant human galectin-4 (Gal4) at 37°C for 1 h with or without agitation. Live bacteria were quantified by colony-forming unit (CFU) enumeration after plating serial dilutions of bacterial suspensions on LB agar plates. Data represent the mean ± SEM, and the *P*-value was calculated using multiple t-tests, *N* = 3 (independent experiments). RPM, revolution per minute. WT (*B*) or *rfaL* mutant (Δ*rfaL*) (*C*) of *S. enterica* serovar Worthington were incubated in LB containing 50 µg/mL BSA, rhGal4 or rhGal9null; live bacteria were quantified by CFU enumeration after plating serial dilutions of bacterial suspensions on LB agar plates. Data represent the mean ± SD, and the *P*-value was calculated using the Mann-Whitney test. NS, not significant. Data are representative of two independent experiments.

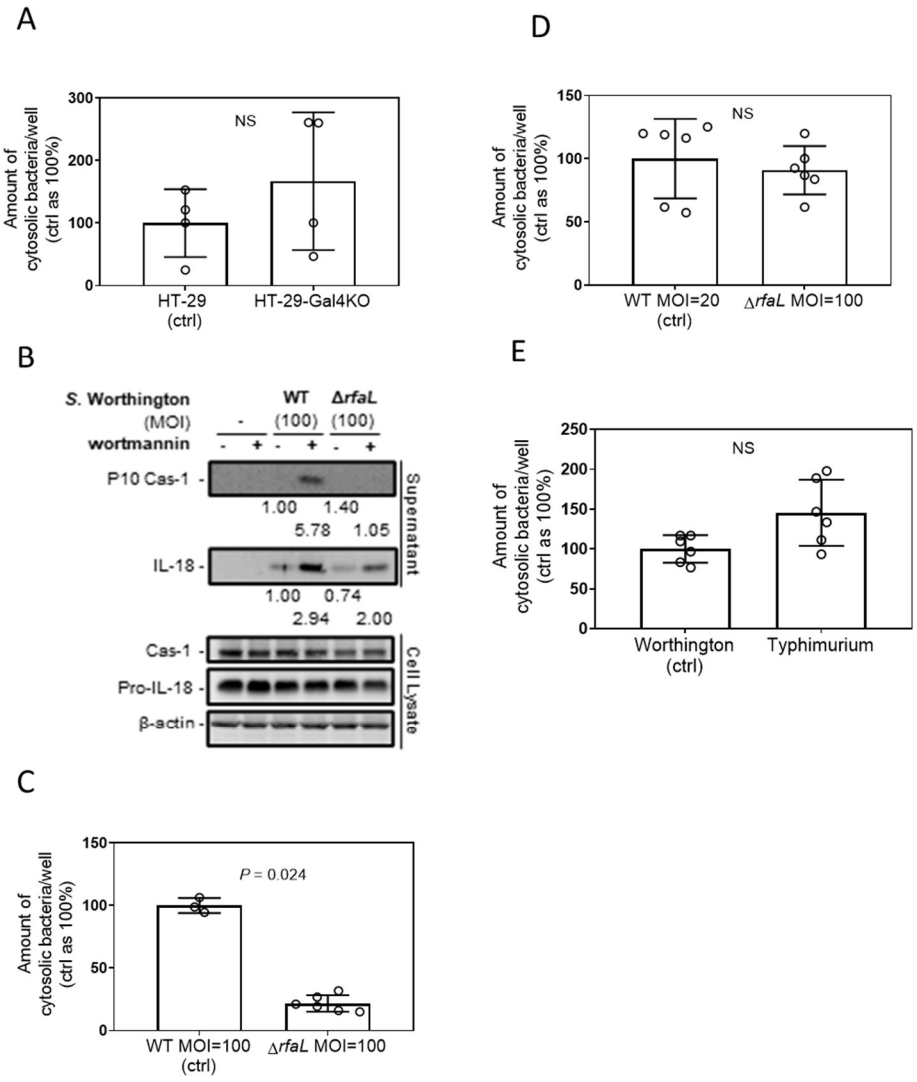


Fig. S10. (A) HT-29 and HT-29-Gal4KO cells treated with wortmannin were infected with *S. enterica* serovar Worthington, MOI = 100. After 4 h of infection, the infected cells were treated with chloroquine for 1 h and the number of cytosolic bacteria were quantified by CFU enumeration. (B) HT-29 cells treated with or without wortmannin were infected with wild type (WT) or *rfaL* deleted (Δ*rfaL*) *S. enterica* serovar Worthington, MOI = 100. Supernatants and cell lysates were collected after 24h post infection and analyzed by immunoblotting with the indicated antibodies. The relative amounts of P10 Cas-1 to Cas-1 and IL-18 to pro-IL-18, as determined by densitometric analysis, are indicated. (C) HT-29 cells treated with wortmannin were infected with WT or Δ*rfaL S. enterica* serovar Worthington, MOI = 100. After 4 h of infection, the infected cells were treated with chloroquine for 1 h and cytosolic bacteria were quantified by CFU enumeration. (D) HT-29 cells treated with wortmannin were infected with WT (MOI = 20) or Δ*rfaL* (MOI = 100) *S. enterica* serovar Worthington. After 4 h of infection, the cells were treated with chloroquine for 1 h and cytosolic bacteria were quantified by CFU enumeration. (E) HT-29 cells treated with wortmannin were infected with *S. enterica* serovar Worthington or *S. enterica* serovar Typhimurium, MOI = 100. After 4 h of infection, the infected cells were treated with chloroquine for 1 h and cytosolic bacteria were quantified by CFU enumeration. Data represent the mean ± SD, and the *P*-value was calculated using the Mann-Whitney test. NS, not significant. Data are representative of two independent experiments.

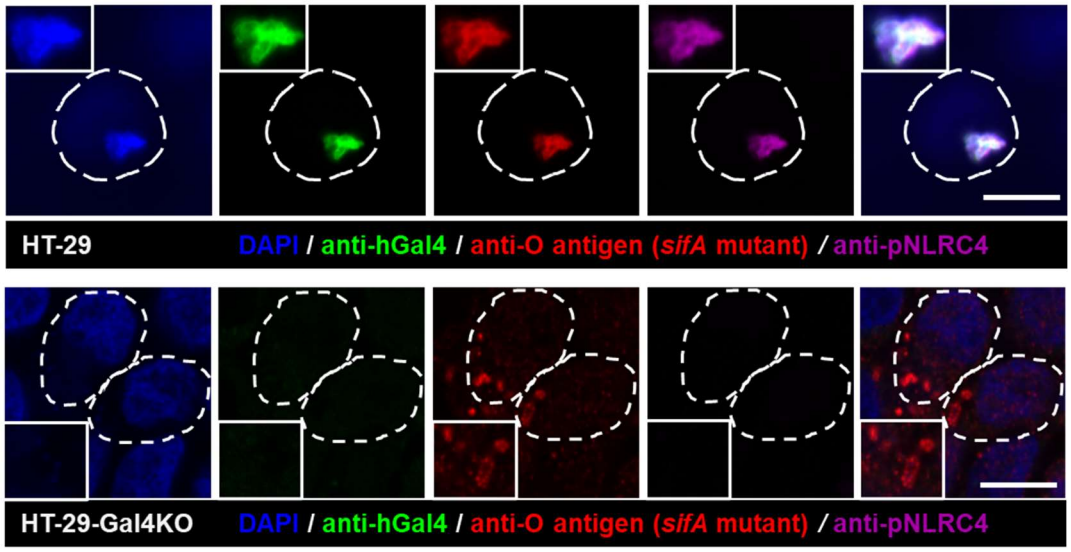


Fig. S11. Galectin-4 colocalizes with *sifA* mutant of *S. enterica* serovar Worthington and phosphor-NLRC4 (pNLRC4) in HT-29. Cells were infected with *sifA* mutant strain, fixed and immunostained to detect galectin-4 (green), *sifA* mutant strain (red), pNLRC4 (purple), and nuclei (blue) at 10–12 h post-infection. Merged signals are shown as white. The borders of the infected cells are outlined with white dashed lines. Scale bars, 10 μm.

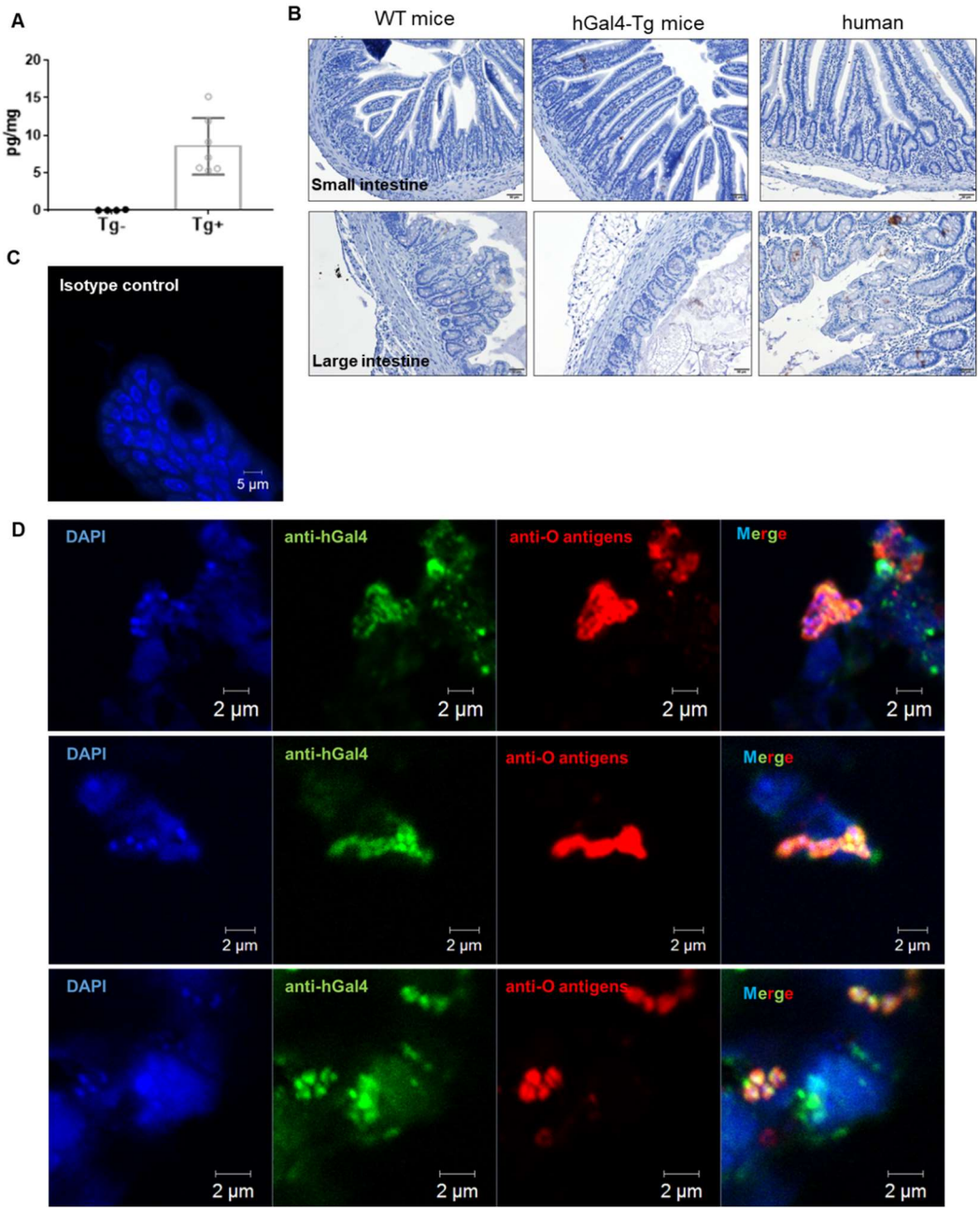


Fig. S12. (A) Human galectin-4 is detected in the feces of hGal4-Tg mice. Feces were collected from wild-type or hGal4-Tg mice and weighed. A total of 200 μL PBS with 50 mM TDG and proteinase inhibitor was added to the fecal samples, which were then vortexed and centrifuged. After centrifugation, the amount of human galectin-4 in the supernatant was determined by ELISA. Data represent the mean ± SD. (B) The images of isotype control staining conducted in Fig. 5B. (C) The image of isotype control staining conducted in Fig. 5C. (D) Confocal images from ileum lumen of hGal4-Tg mice orally received *S. enterica* serovar Worthington after 24 h, DAPI (blue), anti-human galectin-4 (green), anti-O antigens of *Salmonella* (red).

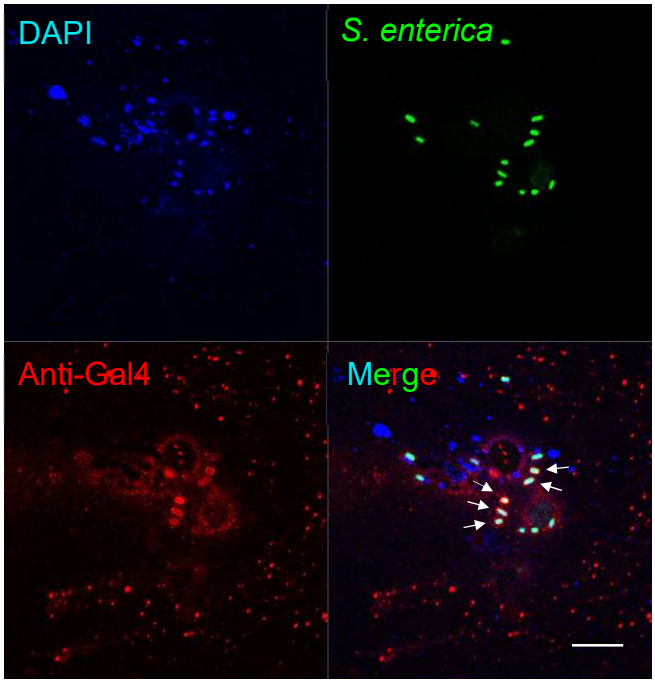
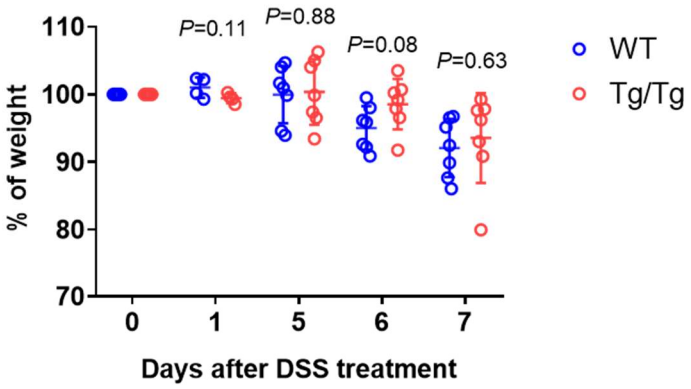


Fig. S13. GFP-expressing *S. enterica* serovar Worthington (green) were injected into the ileal loop of hGal4-Tg mice. Intestinal contents were smeared onto glass slides 3 h after injection, fixed with 4% paraformaldehyde, and stained with anti-human galectin-4 antibody (red) and DAPI (blue). In the merged image, galectin-4-coated bacteria are indicated by white arrows. Scale bar, 10 μm.

A



B

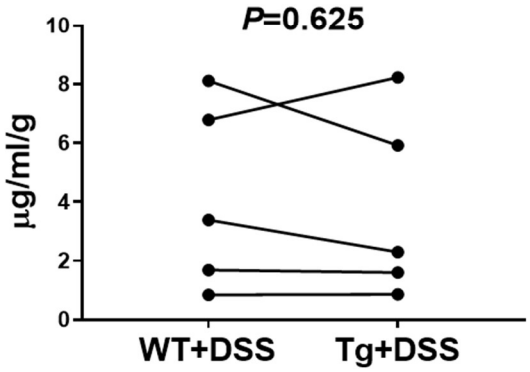


Fig. S14. (A) Weight loss in WT and hGal4-Tg mice after receiving 2.5% DSS in drinking water. Data represent the mean ± SD, and the *P*-value was calculated using multiple t-tests. (B) Mice were treated with 2.5% DSS in their drinking water for 5 days. On day 7, the intestinal barrier permeability of DSS-treated mice was measured by detecting fluorescein isothiocyanate-dextran (FITC) in serum 4 h after oral administration. The *P*-value was calculated using the Wilcoxon matched-pairs signed-rank test.

Table S1

Human galectin-4 CRISPR SgRNA	
For	CATCCCGGGCGGGCTCAACGGTTTT
Rev	CGTTGAGCCCGCCCGGGATGCGGTG
***S.enterica* Worthington *rfaL* KO primer**	
For	CTCATCCCAAACCTATTGTGGAGAAAAGATGCTAACCACAGTGTAGGCTGGAGCTGCTTC
Rev	CTGATACCGTAATAAGTATCAGCGCGTTTTTTTATCTATTATGGGAATTAGCCATGGTCC
***S.enterica* Worthington *rfaL* KO check**	
For	GCTGGCTGGCGCAAAATTTG
Rev	TATTGTGCCATCTCAGGTTG
***S.enterica* Worthington *sifA* KO primer**	
For	GCGCCCGCAGTTGAGATAAAAAGGGTCGATTTAATCGTGTAGGCTGGAGCTGCTTC
Rev	GCCAGGCAAGAGGTTACTCTGTAGGCAAACAGGAAGATGGGAATTAGCCATGGTCC
***S.enterica* Worthington *sifA* KO check**	
For	GCGCCCGCAGTTGAGAT
Rev	GCCTGGCAAGAGGTTACT
Mouse galectin-1 qPCR primer	
For	CTCTCGGGTGGAGTCTTCTG
Rev	GGTTTGAGATTCAGGTTGCTG
Mouse galectin-3 qPCR primer	
For	GCCTACCCCAGTGCTCCT
Rev	GGTCATAGGGCACCGTCA
Mouse galectin-4 qPCR primer	
For	CATGCCTGAGCACTACAAGG
Rev	CGAGGAAGTTGATGGACTGAA
Mouse galectin-7 qPCR primer	
For	ATGAGAATTCGAGGCATGGT
Rev	CACCGCATAGCAGGTTTACA
Mouse galectin-8 qPCR primer	
For	ATCGTGTTCATGGTGCTCAA
Rev	CGTACAGCAGAACATGCCTTC
Mouse galectin-9 qPCR primer	
For	ACCCTACCACCTCGTGGAC
Rev	GACAGGGGCTGCAGAGTTC
Mouse GAPDH qPCR primer	
For	GTATGACTCCACTCACGGCAAAT
Rev	GATGGGCTTCCCGTTGATGA

Movie S1 (separate file). Bacterial chains induced by galectin-4 are derived from cell progeny. Time-lapse imaging of *S. enterica* serovar Worthington cells incubated with 100 μg/mL of recombinant human galectin-4. Time indicated the real time (h:min:sec) on the day of the experiment.

Movie S2 (separate file). Time-lapse imaging of *S. enterica* serovar Worthington cells incubated with 100 μg/mL BSA. Time indicated the real time (h:min:sec) on the day of the experiment.

Movie S3 (separate file). Intracellular galectin-4 in IECs inhibits the motility of cytosolic bacteria (< 20 bacteria per cell). HT-29-EGFP and HT-29-EGFP-Gal4 cells were treated with wortmannin and infected with the RFP-expressing *S. enterica* serovar Worthington (MOI = 50). Time-lapse images were acquired 10 h post-infection using time-lapse microscopy.

Movie S4 (separate file). Intracellular galectin-4 in IECs inhibits the motility of cytosolic bacteria (≥ 20 bacteria per cell). HT-29-EGFP and HT-29-EGFP-Gal4 cells were treated with wortmannin and then infected with the RFP-expressing *S. enterica* serovar Worthington (MOI = 50). Time-lapse images were acquired 10 h post-infection using time-lapse microscopy.

Movie S5 (separate file). Galectin-4 restricts bacterial motility in the intestinal lumen. GFP-expressing *S. enterica* serovar Worthington was injected directly into the ileum of anesthetized wild-type or hGal4-Tg mice. Luminal GFP-expressing *S. enterica* serovar Worthington was tracked individually in intact intestines using two-photon microscopy.

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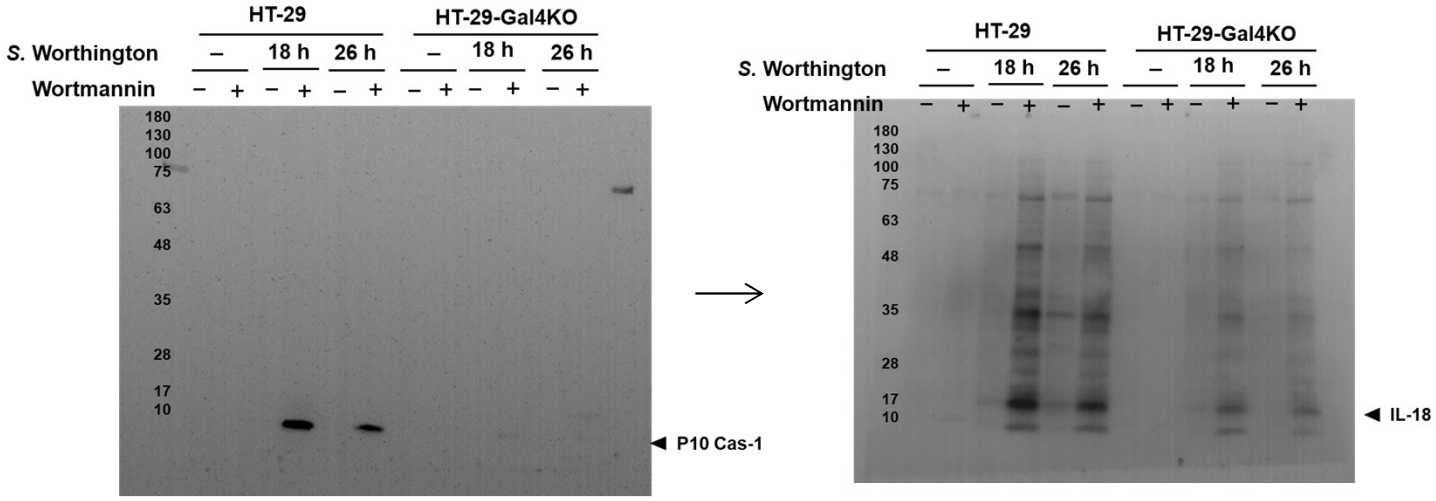
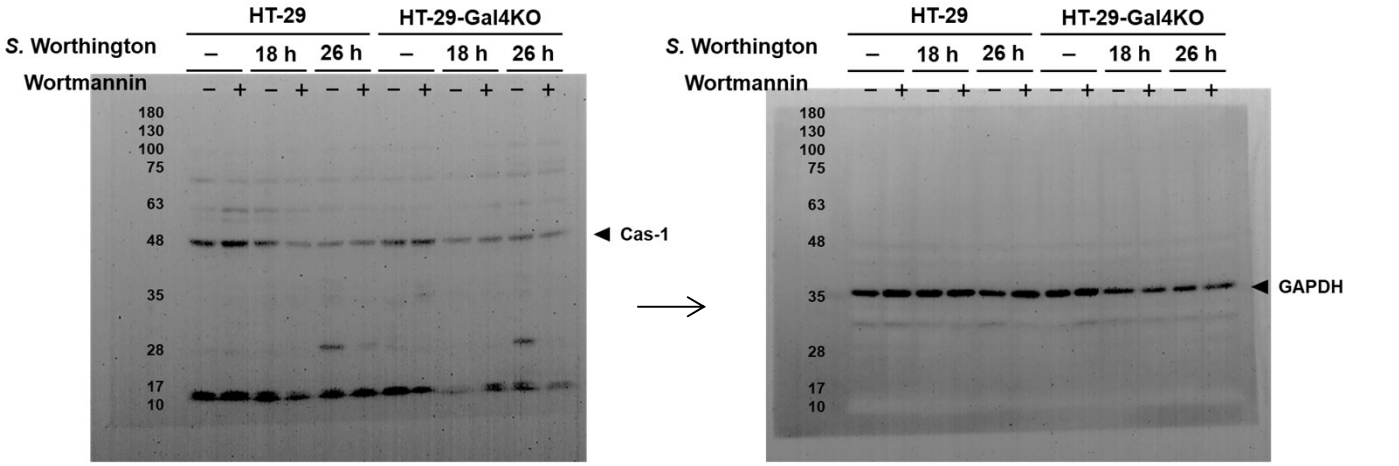
Raw images of Fig. 4A

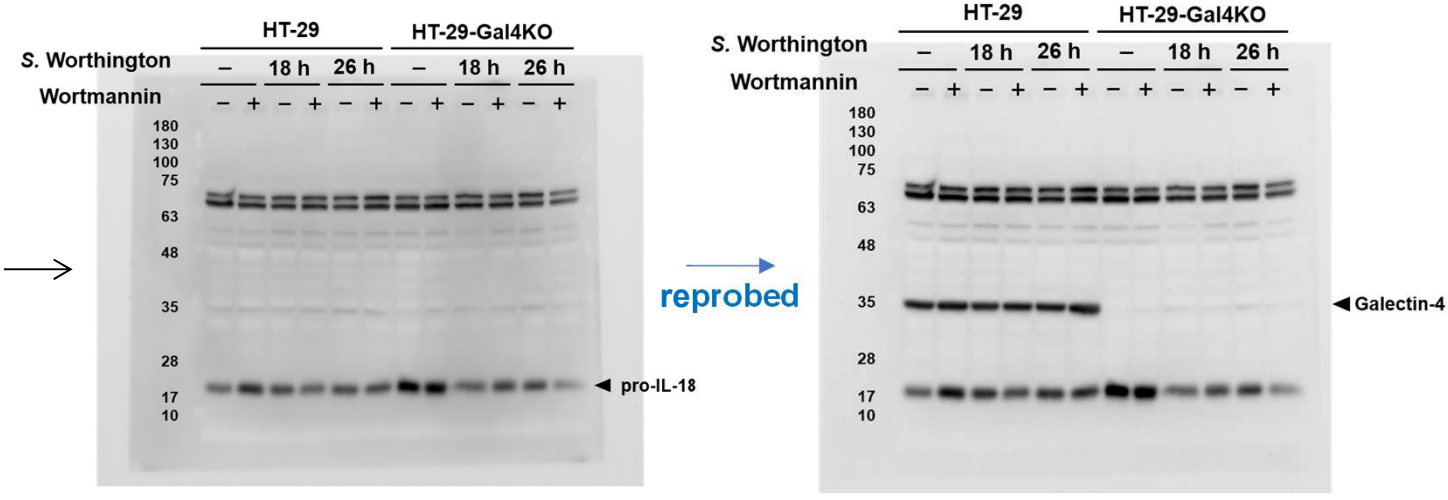
Supernatant

→ Stripped and reprobed



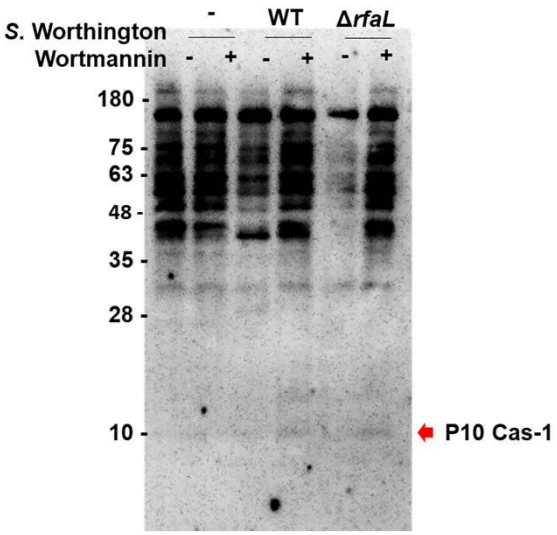
cell lysate

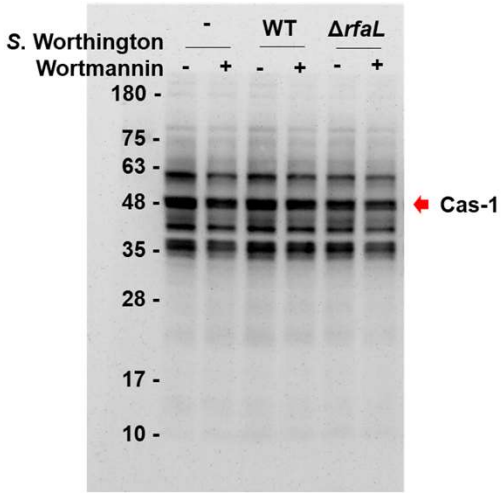


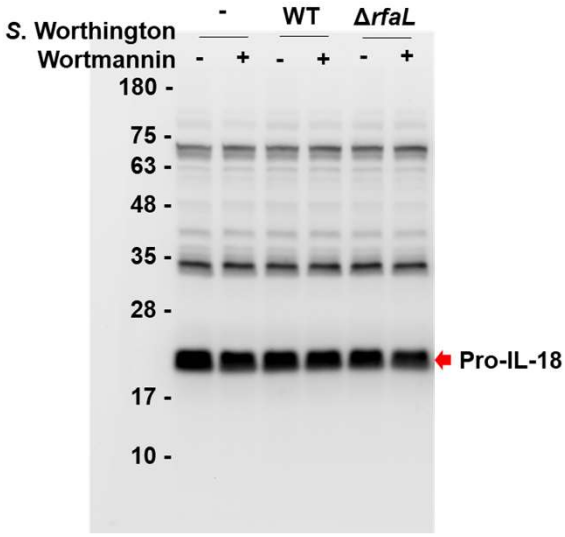


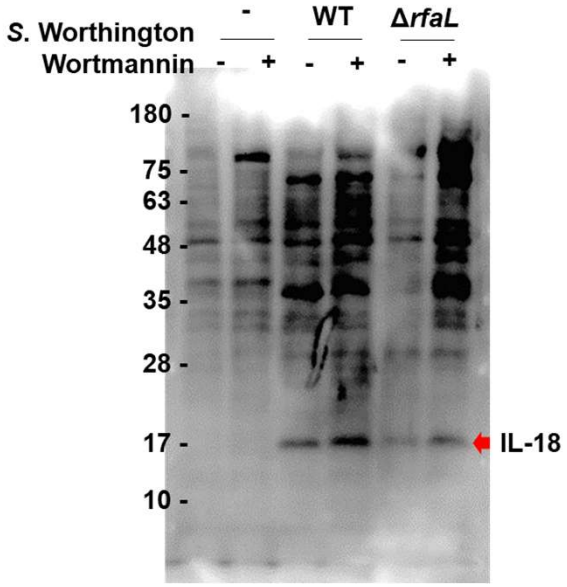
Raw images of Fig. 4B

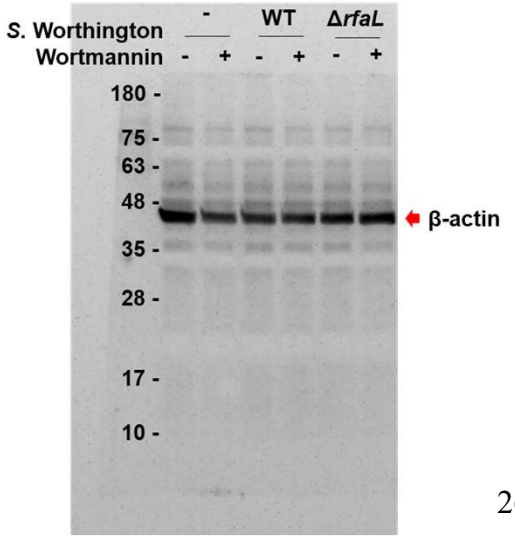
Supernatant ↓ **Stripped and reprobed** **cell lysate**



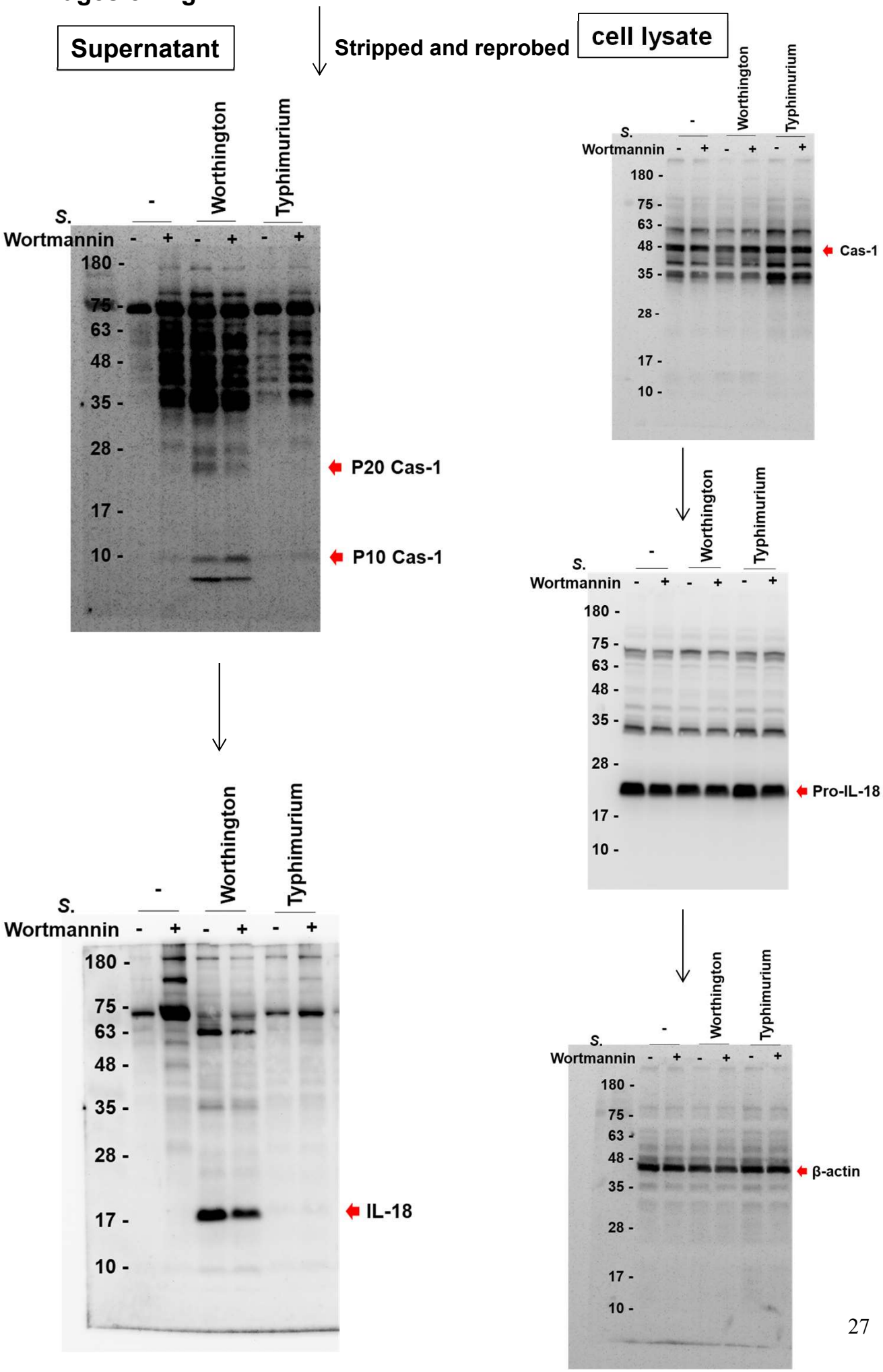








Raw images of Fig. 4C



Raw images of *SI Appendix*, Fig. S10B

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cell lysate