Supporting Information

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SI Materials and Methods

Cell Culture and Transfection. S2 cells were maintained in Schneider's medium (Invitrogen) supplemented with 10% FBS at 26 °C. *Drosomycin*-luciferase reporter plasmid (1) (500 ng) and *Act5c*-Renilla luciferase control plasmid (20 ng) were delivered into 2 mL of S2 cells (1.5×10^6/mL) by calcium phosphate transfection. Transfected cells were stimulated with Spätzle (0.5 μg/mL) for 20 h followed by luciferase assays using the Dual-Glo Luciferase Assay System (Promega), according to the manufacturer's instructions. Stable S2 cells were generated by transfecting cells with 10 μg of the expression construct and 0.5 μg of pBS-puro (E. Izaurralde, Max Planck Institute for Developmental Biology, Tubingen, Germany). After 3 days, transfected cells were selected and maintained in medium containing 5 μg/mL puromycin. S2 cells stably expressing LAMP-GFP were provided by Gudrun Ihrke (Uniformed Services University School of the Health Sciences, Bethesda, MD).

Fly Stocks and Infection. The pWIZ-*mop*-dsRNA plasmid was used to generate transgenic fly lines (2). The expression of mop dsRNA was controlled by the UAS-GAL4 system. The heat-shock GAL4 line (stock No. 1799) and the c564 GAL4 driver (stock No. 6982) were obtained from the Bloomington *Drosophila* Stock Center. Heat-shocked treatment was carried out for 3 days (1 h at 37 °C each time and three times a day). Male progeny from the same cross were pricked a tungsten needle dipped in concentrated *M. luteus* (ATCC No. 4698) or *E. coli*. RNA extracts were prepared from whole flies 4 h (*M. luteus*) or 8 h (*E. coli*) after infection.

Purification of Recombinant Spätzle. Purification and processing of recombinant Spätzle protein were carried out as described (3). Full-length Spätzle proprotein bearing a C-terminal His_6 tag was expressed in Sf9 cells by using the baculovirus system, purified by Ni-NTA agarose (Qiagen), and then incubated with 6 μg/mL of trypsin (Sigma) at 37 °C for 1 h to process pro-Spätzle. The mature C-terminal fragment of 106 amino acid residues was eluted with 200 mM imidazole.

Plasmid Constructs and dsRNAs. *Drosophila mop* is encoded by the *CG9311* gene (NCBI accession number NM_140465). The *mop* and *pelle* cDNA fragments were amplified from total RNA of S2 cells and cloned into pPAC2. *Toll* and *MyD88* ORFs were amplified by PCR using pToll (N. Silverman, University of Massachusetts Medical School, Worcester, MA) and pMT/MyD88-V5 (S. Wasserman, University of California, San Diego, CA) as templates, respectively, and cloned into pPAC2. Human *HD-PTP* was amplified by PCR using the IMAGE clone 6579163 (ATCC) as a template and cloned into the pPAC2 vector. A sequence coding for the HA epitope was joined to the 3′ end of all of the above described coding sequences. The *Drosophila Hrs* coding sequence was amplified by PCR using the EST clone LD30575 as a template (Open Biosystems), and cloned into the pPAC2-3xFlag and pPAC2-HA vectors. The ΔBro1 and ΔPTP *mop* plasmids were generated by PCR using primers within the coding region. The resulting constructs lack the N-terminal 390 or C-terminal 277 amino acid residues, respectively. The *mop* C1728S mutation was generated by *Pfu* polymerase, according to the QuikChange Mutagenesis Kit protocol (Stratagene). Coding sequences of the full-length Cactus or part of Mop (residues 1–400) were cloned into the pHIS bacteria expression plasmid. To generate the pWIZ-*mop*-dsRNA construct containing an inverted repeat of *mop*, a cDNA fragment from nt 1983–3282 of the ORF was cloned into pWIZ as described (2). All constructs were confirmed by sequencing.

Templates for synthesizing dsRNAs were obtained from the Open Biosystem RNAi library and amplified by using a generic T7-GC primer, which contains a specific GC-rich sequence (CCA-CGGGCGGGT) at the 3′ end. DNA templates used to synthesize *mop* or *Hrs* dsRNAs were amplified by PCR from cDNA clones using gene-specific primers containing the GC-rich sequence at the 5′ end. Purified PCR products were used as the template for another round of PCR using the T7-GC primer. The dsRNA sequences of *mop*(i) contains nt 1449–1976, *mop*(ii) contains nt 2775–3280, and *mop*(iii) contains the majority of the 3′ UTR (325 nt). The *Hrs* dsRNA targets nt 1100–1585 of the coding region.

RNAi Screen of *Drosophila* Kinase and Phosphatase Genes. S2/Cact-Luc cells (1.5×10^6 cells/mL) were switched to serum-free medium and 50 μL of cells were plated into a well containing 1 μg of dsRNA in 96-well plates. After a 1-h incubation, complete medium (100 μL) was added to each well. RNAi-treated cells were split into two 96-well plates 3 days after dsRNA treatment, and one plate was stimulated with 0.5 μg of Spätzle for 1 h. Luciferase activities were determined by using the Dual-Glo Luciferase Assay System (Promega) at the end of stimulation. Cactus-Luciferase activities were normalized to the *Renilla* luciferase activities.

Antibody Production. Full-length recombinant Cactus was expressed in BL21(DE3)pLys *E. coli*. Bacteria were frozen at −80 °C before being lysed in native lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl at pH 8.0). The insoluble fraction, which contained the majority of Cactus, was washed five times with native lysis buffer supplemented with 2% Triton X-100 and then boiled in SDS sample buffer. Protein extracts were resolved by SDS/PAGE. Recombinant Cactus protein was excised from the gel and used to immunize guinea pigs. The N-terminal fragment of mop containing 400 amino acid residues was expressed in BL21(DE3)pLys *E. coli* and purified with Ni-NTA agarose (Qiagen) according to manufacturer's instructions. Purified protein was used to immunize rabbits. The antigen was conjugated onto NHS-activated Sepharose (GE Healthcare) to generate an affinity column, which was used to purify anti-Mop antibodies.

Immunofluorescence Staining. S2 cells were fixed in 4% paraformaldehyde for 10 min and then permeablized by incubating with 0.2% Triton X-100 in PBS for 5 min at room temperature. After blocking, cells were incubated overnight with the appropriate antibodies diluted in PBS containing 10% horse serum and 0.2% Triton X-100. Antibodies used were specific for the Bro1 domain of Mop, the Flag epitope (M2; Sigma-Aldrich), the HA epitope (HA.11; Covance), *Drosophila* KDELR (KR-10; Calbiochem), *Drosophila* Golgi (7H6D7C2; Calbiochem), *Drosophila* Rab5 (ab31261; Abcam), and mitochondrial complex V subunit α (15H4C4; MitoSciences). Fluorescein- or rhodamine red X-conjugated anti-mouse IgG or anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch.

Real-Time Quantitative PCR. RNAi-treated cells were stimulated with 1.0 μg/mL Spätzle for 6 h or 10 ng/mL LPS (*E. coli*, serotype 055:B5; Sigma-Aldrich) for 3 h. RNA was isolated by using the Absolutely RNA Miniprep Kit (Stratagene), and 300 ng of RNA was reverse-transcribed with the iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions. Real-time PCR was carried out by using the Mx3000P QPCR System (Stratagene) with SYBR Green (Bio-Rad). Primer pairs used for detection are:

TCCGTGAGAACCTTTTCCAATATG/CCAGGACCAC-CAGCATCAG (*Drs*),
TCCACTGTCGCCCGATCC/CTTGGGTTGAAACTTCC-TACTTGC (*IM1*),
GCGTTGGTCAGCACACTC/CAGTTGCGGCGA-CATTGG (*CecA1*)
AGGTTCCTTAACCTCCAATC/CATGACCAG-CATTGTTGTAG (*AttA*), and
CTTGATTCTGCTGGAGGAGGAG/CATCTTG-TAGTTGGTGTTGTTGTTG (*Act5C*).

Immunoblot and Immunoprecipitation Assays. RNAi cells were stimulated with 0.5 μg/mL Spätzle for 20 min, and then lysed in protein extraction buffer (1× TBS buffer, 0.5% Igepal CA-630, 1× EDTA-free protease inhibitor mixture, and 1 mM DTT). After incubation on ice for 5 min, lysates were spun at 14,000 × *g* for 10 min. Cleared supernatant fractions (30 μg) were resolved by SDS/PAGE and transferred onto PVDF membranes (Bio-Rad), which were then probed with antibodies and detected with Chemiluminescent HRP Substrate (Millipore). For immunoprecipitation assays, expression plasmids for dHrs-3xFlag (4 μg), Toll-HA (4 μg), and Mop-HA (4 μg) were transfected into S2 cells. Cells were lysed in protein extraction buffer at 48 h after transfection. Lysate was incubated on ice for 5 min, spun at 14,000 × *g* for 10 min, and filtered through a 0.22-μm membrane to remove lipids. Anti-Flag resin or normal IgG was added to protein extracts. After 2 h of incubation at 4 °C, protein A/G agarose (Pierce) was added to the IgG control and mixed overnight at 4 °C. The immunoprecipitates were washed 4 times with 1× TBS buffer containing 0.5% Igepal CA-630, eluted by SDS sample buffer, and analyzed by immunoblotting with antibodies specific to HA or Flag epitope.

1. Tauszig S, Jouanguy E, Hoffmann JA, Imler JL (2000) Toll-related receptors and the control of antimicrobial peptide expression in Drosophila. *Proc Natl Acad Sci USA* 97: 10520–10525.
2. Lee YS, Carthew RW (2003) Making a better RNAi vector for Drosophila: use of intron spacers. *Methods* 30:322–329
3. Weber AN, et al. (2003) Binding of the Drosophila cytokine Spatzle to Toll is direct and establishes signaling. *Nat Immunol* 4:794–800.

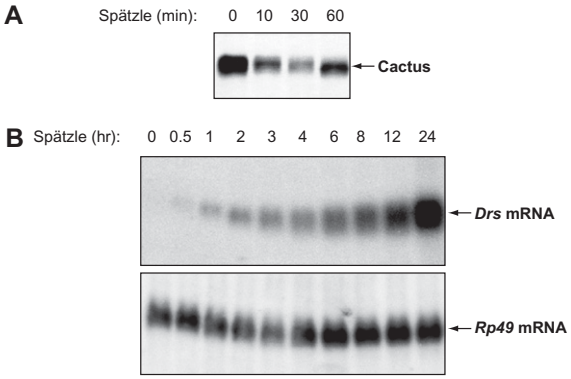

Fig. S1. Recombinant Spätzle protein activates the Toll pathway. (*A*) S2 cells were stimulated with mature Spätzle protein for 0, 10, 30, or 60 min. Protein extracts (30-μg aliquots) were resolved by SDS/PAGE, and the level of Cactus protein was determined by immunoblotting with a Cactus-specific antibody. (*B*) RNA was isolated from S2 cells stimulated with mature Spätzle protein at different time points and analyzed by Northern blotting. Activation of Toll signaling was monitored by the induction of *Drosomycin* (*Drs*). The levels of *Rp49* were used as a loading control.

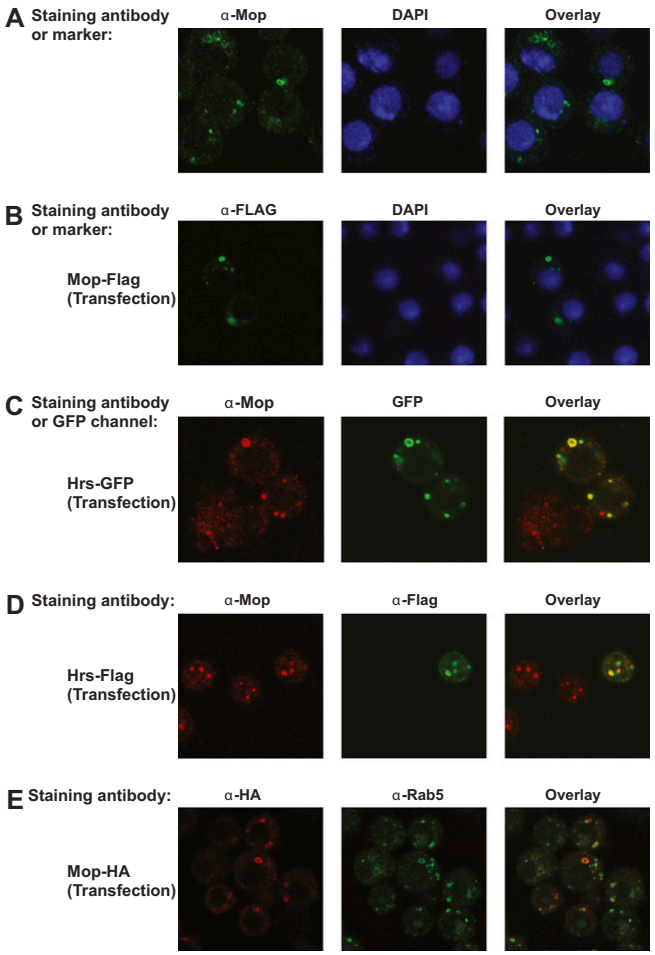

Fig. S2. Mop is localized to endosomes. (*A*) S2 cells were stained with an antibody against endogenous Mop (anti-Mop, green). Nuclei were stained with DAPI (blue). The overlay image indicates that Mop is present in the cytoplasm. The images were obtained by confocal microscopy. (*B*) S2 cells were transfected with a plasmid expressing Mop-Flag, which was then visualized with anti-Flag antibody (green). Nuclei were stained with DAPI (blue). (*C*) S2 cells were transfected with a Hrs-GFP expression plasmid. Mop was visualized with an anti-Mop antibody (red). The yellow signals in the overlay images indicate colocalization of Mop and Hrs. (*D*) Similar to *C*, except a Hrs-3xFlag expression plasmid was used in transfection, and the protein was detected with an anti-Flag antibody (green). (*E*) S2 cells stably expressing HA-tagged Mop were stained anti-Rab5 (green) and anti-HA (red) antibodies. The yellow signals in the overlay image represent colocalization of Mop and Rab5.

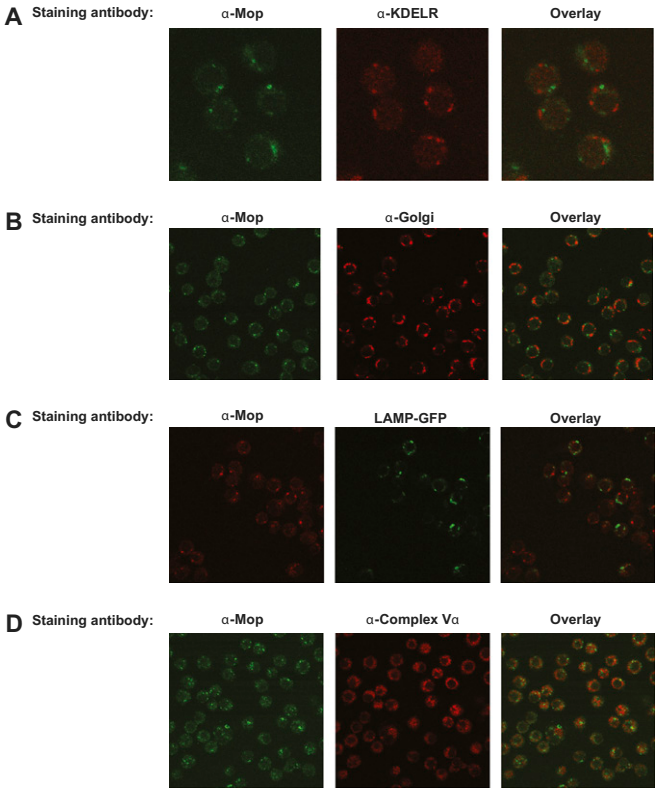

Fig. S3. Mop distribution and organellar markers. (*A*) S2 cells were stained with anti-Mop (green) and anti-KDELR (red) antibodies and imaged by confocal microscopy. (*B*) Antibodies specific to Mop (green) and Golgi (red) were used to stain S2 cells and imaged by confocal microscopy. (*C*) S2 cells stably expressing a GFP-tagged lysosomal marker (LAMP-GFP) were stained with anti-Mop antibody (red). Images were analyzed by confocal microscopy. (*D*) S2 cells were stained with antibodies against Mop (green) and the α subunit of the mitochondrial complex V (red) and analyzed by confocal microscopy.

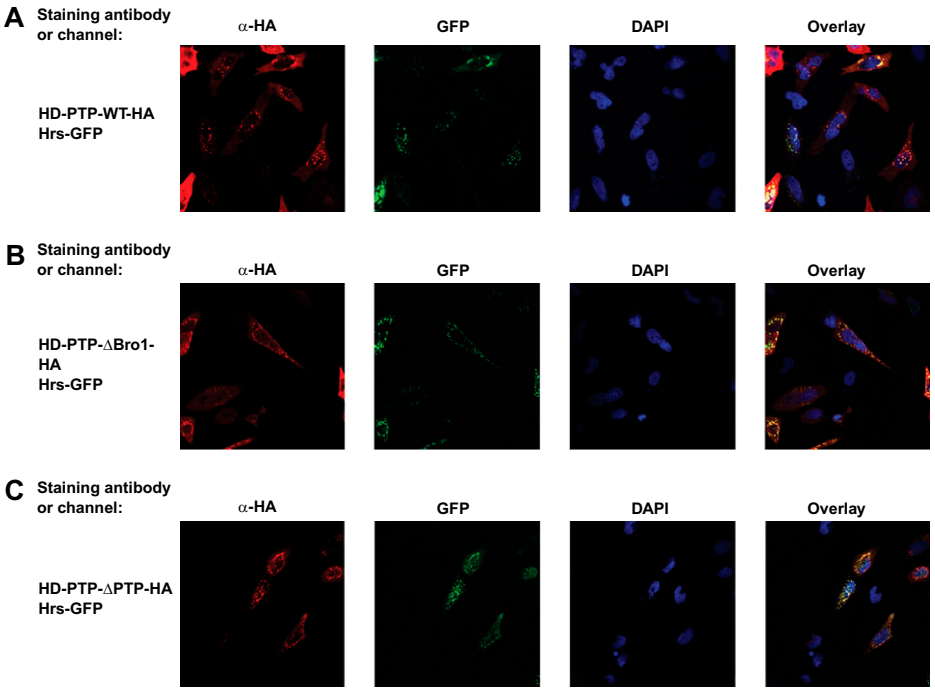

Fig. S4. Neither the Bro1 nor PTP domain is required for the human HD-PTP to localize to endosomes. HeLa cells were transfected with plasmids expressing HA-tagged HD-PTP proteins [wild type (*A*), ΔBro1 (*B*), or ΔPTP (*C*)] and Hrs-GFP. Transfected cells were fixed and stained with antibodies specific to the HA epitope and DAPI for the nucleus. Wild-type and mutant HD-PTP proteins were present to endosomes and colocalized with human Hrs-GFP.