Supporting Information

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SI Experimental Procedures

Cell Culture and Preparation of Primary Macrophages. RAW264.7 and 293T cells (ATCC) were maintained in DMEM containing 10% FBS, 2 mM glutamine, and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin). Bone marrow-derived macrophages (BMDMs) were generated from bone marrow progenitors collected from both femur and tibia of C57BL/6 mice and 5×10^6 cells were differentiated in RPMI medium 1640 containing 10% FBS and 20 ng/mL M-CSF (R&D Systems) in a six-well plate for 72 h. Procedures on animals were approved by the Animal Care and Use Committee of the National Cancer Institute. All cell-culture media and supplements were obtained from Invitrogen. Cells were cultured in a humidified atmosphere at 37 °C in 5% CO_2.

Preparation of Cells Expressing GFP-LC3. RAW cells and BMDMs expressing GFP-light chain 3 (LC3) were prepared by retrovirus-mediating gene transfer. The retroviral expression vector pMYs-IRES-GFP-LC3 was constructed from a backbone vector pMYs-IRES-GFP (1). LC3A cDNA was transferred into pEGFP-C1 (Clontech) to produce GFP-LC3. $LC3^{G120A}$ and $LC3^{K51A}$ cDNAs were prepared with QuikChange II site-directed mutagenesis kit (Stratagene). For retrovirus production, 293T (1.8×10^6) cells growing in 6-cm dishes for 24 h were transfected with a DNA mix containing 3 μg of each of pMYs-IRES-GFP-LC3, pVPack-GP, and pVPack-VSV-G (Stratagene) using Lipofectamine 2000 (Invitrogen), and after 48 h the supernatant containing the retroviral particles was collected. For retroviral infection, RAW cells (1×10^5) or BMDMs (1×10^6) growing in a six-well plate for 24 h were incubated with retroviral supernatant and 10 μg/mL DEAE-dextran for further 48 h. RAW cells transduced with GFP-LC3 were sorted with a FACSAria (BD) to enrich GFP^+ cells.

Autophagy Assay. Cells (RAW cells, 2.5×10^5; BMDMs, 2×10^6) growing in a six-well plate for 24 h were treated with LPS (0.1 or 1 μg/mL, 0111:B4; Sigma) or *Escherichia coli* (0.5 or 5×10^7, K12 BioParticles; Invitrogen) for the indicated times, in the presence or absence of PepA and E64d (10 μg/mL of each), or polymyxin B (PMB) (10 μg/mL), or 3-MA (0.2 mM), or wortmannin (1 μM), or SB202190 (0.5 μM), or SB203580 (0.5 μM), or *N*-acetyl-L-cysteine (10 mM), or SB202474 (0.5 μM), or PD169316 (0.5 μM), or JNK inh. II (0.5 μM). All chemicals were obtained from Sigma except for SB202474, PD169316, and JNK inh. II, which were purchased from Calbiochem. Cell extracts were prepared in extraction buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 2% Triton X-100, a protein inhibitor mixture (Roche)] and a portion (20 μg) was subjected to immunoblot analysis with appropriate antibodies. To quantify $GFP\text{-}LC3^+$ dot formation, live cell images of GFP-LC3 expressing cells were acquired from three different fields on a fluorescent microscope (IX81; Olympus) using Slidebook software v4.1.0.16 (Intelligent Imaging Innovations). Using the ImageJ program, 100 to 500 cells were counted and the percentage of cells with $GFP\text{-}LC3^+$ dots was calculated.

RNAi. All gene knockdown assays were performed using Stealth RNAi siRNA (Invitrogen). As a nonspecific control, stealth $RNAi^-$ control duplexes were used. Cells (RAW cells, 2.5×10^5; BMDMs, 2×10^6) growing in a six-well plate were transfected with siRNAs (10 nM) for 32 h using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. Small interfering RNAs are used for ATG5 (#1, cat. no. MSS247019; #2, MSS247021), ATG7 (#1, cat. no. MSS232487; #2, MSS232488), p62 (#1, cat. no. MSS207329; #2, MSS207330), MyD88 (#1, cat. no. MSS275879; #2, MSS275880), IRAK4 (#1, cat. no. MSS218660; #2, MSS218662), TRAF6 (#1, cat. no. MSS212085; #2, MSS212086), TRIF (#1, cat. no. MSS200702; #2, MSS200704), and Nrf2 (#1, cat. no. MSS207017; #2, MSS207018).

Confocal Imaging. Cells (RAW cells, 1×10^5; BMDMs, 1×10^6) cultured on collagen-coated coverslips (BD) were fixed with 4% paraformaldehyde in PBS and subsequently permeabilized with 0.1% Triton X-100. When anti-LC3 antibody was used, cells were permeabilized with methanol. After treatment with 10% goat serum, the cells were incubated with primary antibodies, anti-LC3 (PD014; MBL), anti-p62 (PM045; MBL) or anti-ubiquitin (FK2; MBL) followed by appropriate secondary antibodies conjugated with Alexa dyes (Invitrogen). The coverslips were mounted on slides using DAPI-containing mounting solution (Vector Laboratories) and digital images were acquired with a Zeiss LSM 510 confocal system using a 63× 1.4NA Plan-Apochromat oil-immersion objective.

Postembedding Immune Electron Microscopy. RAW cells were fixed with 4% formaldehyde-0.05% glutaraldehyde in PBS for 2 h at 4 °C, dehydrated with ice-cold graded ethanol series and embedded in LR White resin. After thin sections were blocked in blocking buffer (EMS), the sections were incubated with primary antibody anti-p62 (PM045; MBL) followed by immunogold-conjugated secondary antibody (EMS). Grids were stained in uranyl acetate, followed by lead citrate to enhance contrast. Sections were examined in an H7600 electron microscope (Hitachi), and digital images were taken with a 4K camera (AMT).

Immunoblot Analysis. Immunoblot analysis was performed as previously described (2). Cellular proteins were separated into detergent-soluble and -insoluble fractions with the 2% Triton X-100 buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 2% Triton X-100, a protein inhibitor mixture (Roche)]. The insoluble fractions were solubilized in the buffer with 1% SDS. The detergent-soluble (20 μg) and -insoluble (10 μg) fractions were subjected to immunoblot analysis. Both α-tubulin and lamin A/C were used as fractionation controls of the detergent-soluble and -insoluble fractions, respectively. The following antibodies/antisera were used: mouse monoclonal to LC3 (51-11; MBL), inducible NOS (54; BD), β-actin (AC15; Sigma), ubiquitin (1B3; MBL), α-tubulin (DM1A; Sigma), lamin A/C (clone 14; Millipore), or TRAF6 (D-10; Santa Cruz Biotechnology); rabbit polyclonal to ATG12 (#2011; Cell Signaling Technology), ATG7 (PM039; MBL), p62 (PM045; MBL), MyD88 (ab2064; Abcam), IRAK4 (ab13685; Abcam), or Nrf-2 (C-20; Santa Cruz Biotechnology).

Real-Time RT-PCR. RNA preparation, RT reaction and real-time PCR were performed as previously described (2). PCR primers for p62 (cat. no. 04692306001), LC3B (cat. no. 04685059001), IL-6 (cat. no. 04685032001), NAD(P)H:quinone oxidoreductase (cat. no. 04689097001), peroxiredoxin 1 (cat. no. 04692144001), Nrf2 (cat. no. 04685008001), and 18S rRNA (cat. no. 04688520001) as an internal control were designed using Roche Universal Probe Library. Fold-changes were calculated by using the ΔΔCT method with the untreated sample as a calibrator.

Statistical Analysis. The experiments were performed in triplicate and repeated at least twice. Figures show the results from one representative experiment. Data represent mean values and SD from triplicate determination were calculated and compared using the Student's unpaired *t* test. *P* values less than 0.005 were considered significant.

1. Kitamura T, et al. (2003) Retrovirus-mediated gene transfer and expression cloning: Powerful tools in functional genomics. *Exp Hematol* 31:1007–1014.

2. Fujita K, Janz S (2007) Attenuation of WNT signaling by DKK-1 and -2 regulates BMP2-induced osteoblast differentiation and expression of OPG, RANKL and M-CSF. *Mol Cancer* 6:71.

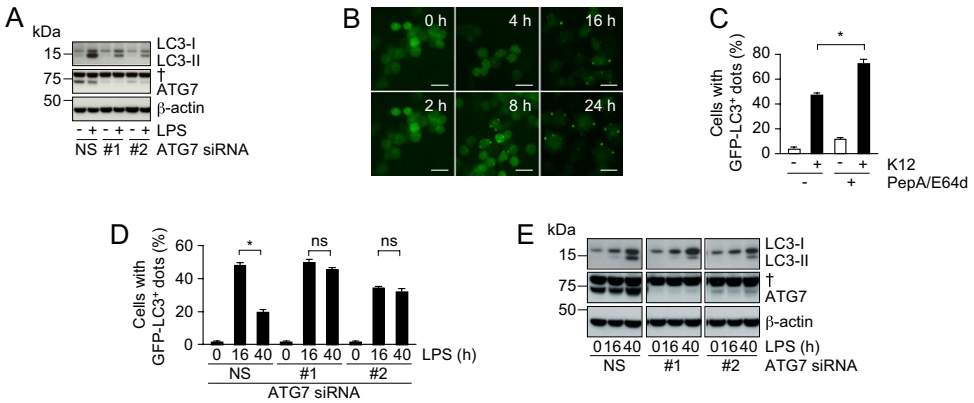

Fig. S1. Formation of Toll-like receptor-4 (TLR4)-mediated LC3$^+$ dots occurs independently of classic autophagic machinery. (*A*) Immunoblots of extracts of RAW cells transfected with ATG7 siRNAs (NS, #1, or #2) and treated with LPS for 24 h probed for the indicated proteins. † Represents nonspecific bands. (*B*) Fluorescent images of live GFP-LC3–expressing RAW cells treated with LPS at the indicated times. (Scale bars, 20 μm.) (*C*) Percentage of cells with GFP-LC3$^+$ dots of RAW cells expressing GFP-LC3 treated with K12 (5×10^6) in the presence or absence of PepA/E64d. (*D* and *E*) GFP-LC3 expressing RAW (*D*) or RAW cells transfected with ATG7 siRNAs (*E*) were treated with LPS at the indicated times. Percentage of cells with GFP-LC3$^+$ dots (*D*) and immunoblots of cell extracts probed for the indicated proteins (*E*) are shown. The results shown are means ± SD; *$P < 0.005$; ns, not significant.

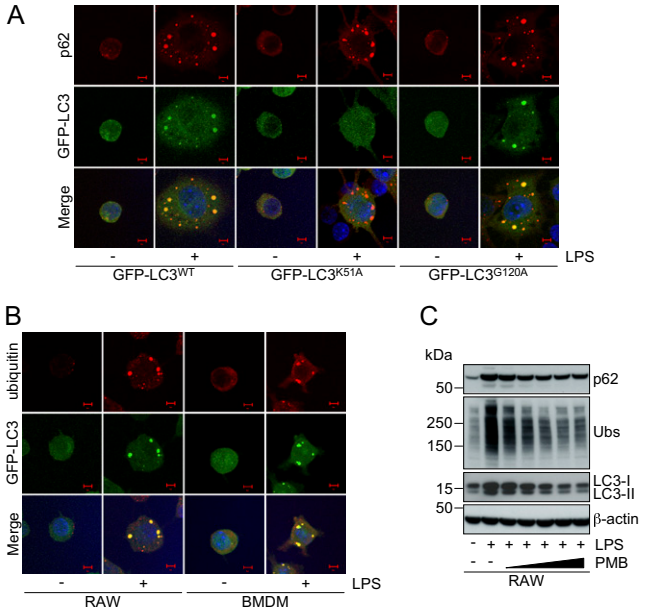

Fig. S2. p62 is a component of LPS-induced aggresome-like induced structures (ALIS). (*A*) Confocal images of cellular localization of p62 in RAW cells. Cells expressing GFP-LC3^{WT}, -LC3^{K51A}, or -LC3^{G120A} were treated with LPS and immunostained for p62. (Scale bars, 5 μm.) (*B*) Confocal images of cellular localization of the ubiquitin in RAW cells (*Left*) and BMDMs (*Right*) expressing GFP-LC3 stimulated with LPS. (Scale bars, 5 μm.) (*C*) Immunoblots of the detergent-soluble fractions from RAW cells treated with LPS along with a series of threefold dilutions of PMB probed for the indicated proteins. Ubs, ubiquitinated proteins.

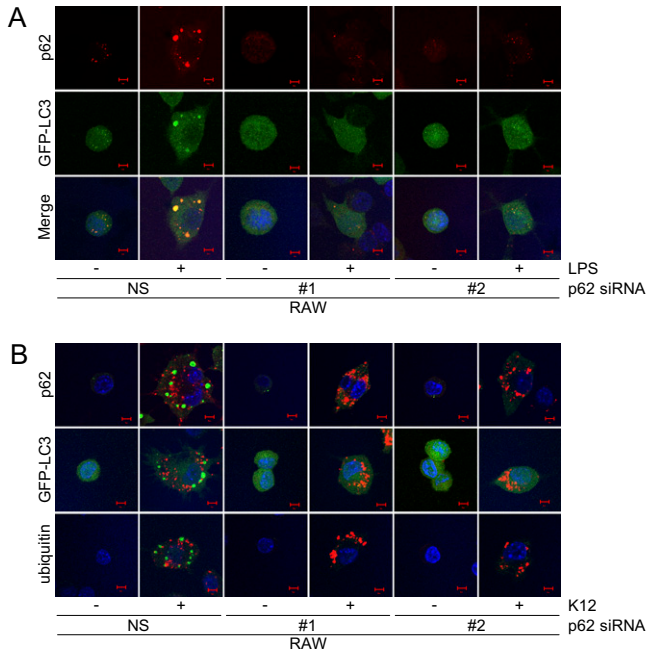

Fig. S3. p62 is required for TLR4-mediated ALIS formation. (*A* and *B*) GFP-LC3 expressing RAW (*A*, *B Middle*) and RAW cells (*B*, *Top* and *B*, *Bottom*) transfected with p62 siRNAs (NS, #1, or #2) were treated with 1 μg/mL LPS (*A*) or 5×10^6 Texas-Red labeled K12 (*B*) for 16 h, and immunostained for the indicated proteins. Confocal images of cellular localization of p62, LC3, and ubiquitin are shown. (Scale bars, 5 μm.)

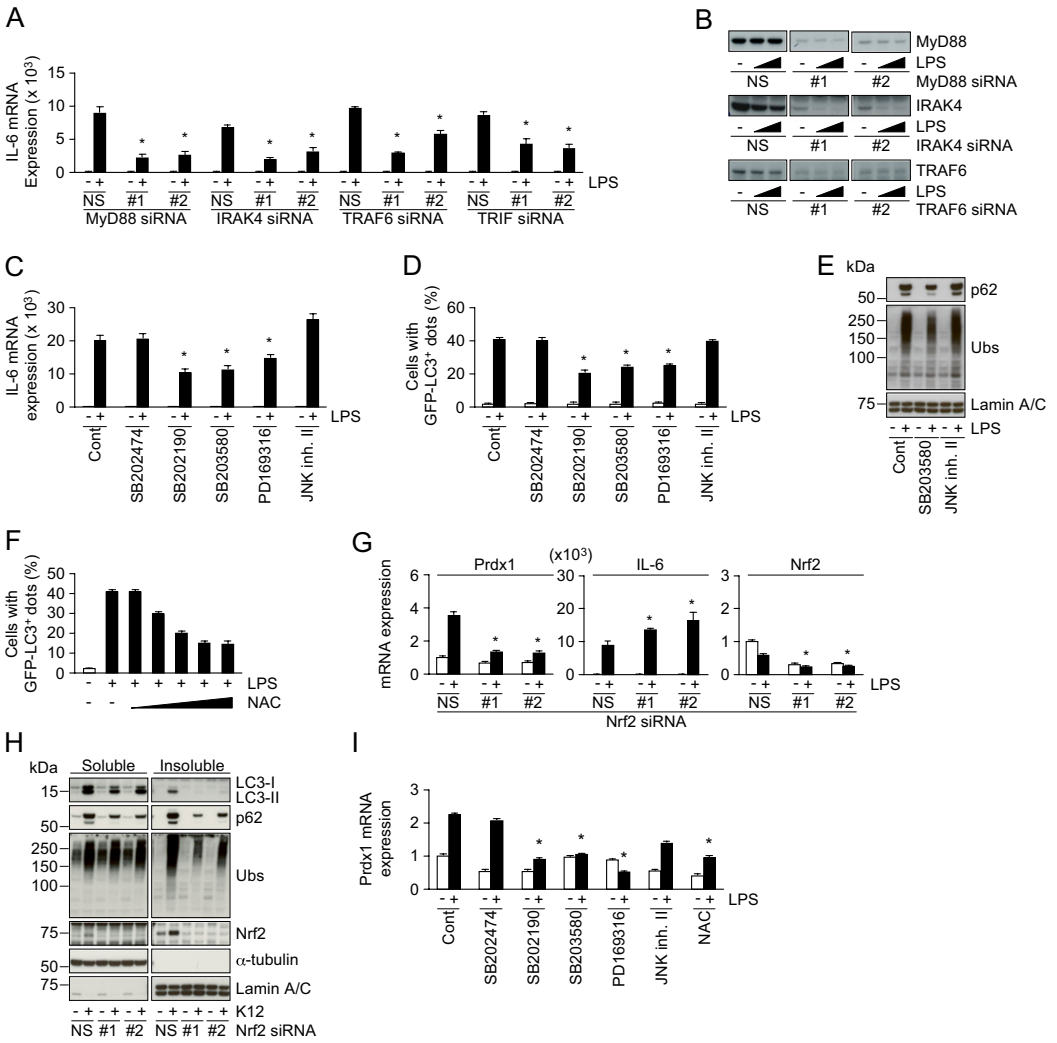

Fig. S4. MyD88-dependent activation of p38 and Nrf2 is required for the induction of p62. (*A* and *B*) Cells transfected with siRNAs (NS, #1, or #2) for the indicated molecules were stimulated with LPS (1 μg/mL, 16 h). The levels of IL-6 mRNA (*A*) and immunoblots of extracts from RAW cells (*B*) probed for the indicated proteins are shown. (*C*–*F*) Cells incubated without (Cont) or with the indicated inhibitors (*C*–*E*), or in the presence of a series of threefold dilutions of *N*-acetyl-L-cysteine (*F*), were stimulated with LPS. The levels of IL-6 mRNA (*C*), percentage of cells with GFP-LC3$^+$ dots (*D* and *F*), immunoblots of the detergent-insoluble fractions probed for the indicated proteins (*E*) are shown. Ubs, ubiquitinated proteins. (*G* and *H*) Cells transfected with Nrf2 siRNAs (NS, #1, or #2) were stimulated with either LPS (*G*) or K12 (*H*). The levels of mRNAs of Prdx1, IL-6, and Nrf2 (*G*) and immunoblots of the detergent-soluble and -insoluble fractions probed for the indicated proteins (*H*) are shown. (*I*) RAW cells incubated without (Cont) or with the indicated inhibitors were stimulated with LPS. The levels of Prdx1 mRNA are shown. The results shown are means ± SD; *statistical significance compared with NS or Cont ($P < 0.005$).