Supporting Information

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SI Materials and Methods

Fly Genetics. Approximately 500 white-eyed flies were selected, and independent lines were established for the purpose of analyzing the *p38b* gene. Independent lines containing KG05418, KG05834, or EY11791 excisions were established (around 200 each) to characterize deficiencies in *p38a*, *p38c*, and *dMK2*, respectively. Genomic DNA was isolated from each line and the expected deletions were identified by PCR with specific primer pairs. The expected deficiency lines were validated by sequence analyses. The $p38a^{13}$ allele is a 1.4-kb deletion that uncovers the promoter region and 5′ portion of the *p38a* transcript. The $p38b^{156A}$ allele contains a 900-bp deletion, including the promoter and 5′ portion of *p38b*; $p38c^{7}$ contains a 2.1-kb deletion in which the entire *p38c* gene and 5′ portion of CG31133 were deleted. The promoter region and 5′ portion of the *dMK2* transcript, including the first two exons, are deleted in line $dMK2^{5B}$. It was further confirmed with RT-PCR that $p38a^{13}$, $p38b^{156A}$, $p38c^{7}$, and $dMK2^{5B}$ are null alleles of *p38a*, *p38b*, *p38c*, and *dMK2*, respectively (Fig S1 *D* and *E*). During the phenotype analysis of these mutants, mutant $p38a^{1}$ was reported (1). RT-PCR results indicate that $p38a^{1}$ is a *p38a* and *p38c* double mutant (Fig S1*D*).

The following fly stocks were used:

y^{1} w^{67c23} (Bloomington stock 6599)

y^{1} *w**; ry^{506} Sb^{1} *P{ry[+t7.2]=Delta2-3}99B/TM6* (Bloomington stock 3664)

y^{1} w^{67c23}; ry^{506}*P{*$y^{+mDint2}$ *w[BR.E.BR]=SUPor-P}*^{KG05834} (Bloomington stock 14126)

y^{1}; ry^{506}*P{*$y^{+mDint2}$ *w[BR.E.BR]=SUPor-P}CG6178*^{KG05318}(Bloomington stock 13992)

y^{1} w^{67c23}; *P{*$y^{+mDint2}$ *w[BR.E.BR]=SUPor-P}p38b*^{KG01337}; ry^{506} (Bloomington stock 14364)

y^{1} w^{67c23} *P{w[+mC] y[+mDint2]=EPgy2}*^{EY11791} (Bloomington stock 20697)

da-Gal4 (Bloomington stock 5460)

cn^{1} bw^{1} Hsf^{4}(Bloomington stock 5489)

cn^{1} bw^{1} Hsf^{4}; *P{w[+mC]=Hsf[+t8]}1/TM3,* Sb^{1} Ser^{1}(Bloomington stock 5490)

y^{1} w^{67c23}; *P{y[+mDint2] w[BR.E.BR]=SUPor-P}Hsp27*^{KG03935} ry^{506}(Bloomington stock 13268)

y^{1} w^{67c23}; $p38a^{1}$

y^{1} w^{67c23}; $p38a^{13}$

y^{1} w^{67c23}; $p38c^{7}$

y^{1} w^{67c23}; $p38b^{156A}$

y^{1} w^{67c23} $dMK2^{5B}$

y^{1} w^{67c23}; $p38b^{156A}$;$p38a^{1}$/*SM5-TM6B*

y^{1} w^{67c23};$p38b^{156A}$; $p38a^{13}$/*SM5-TM6B*

w UAS-p38b[w+]

imd^{1}; spz^{RPF7}

Tl^{10b} $mwh^{1}e^{1}$/*TM3* Ser^{1} Sb^{1}; *OR66*

y^{1} w^{67c23}; *UAS-hsp27.II*

Tl^{r632} *ca/TM6C*

drosom-lacZ; $Tl^{1\text{-}RXA}$/*TM6C*

Tl^{9QURE} *e ca/TM3 Ser*

dipt-lacZ; b pr imd

Complementary DNA Microarray Analysis. Complementary DNA microarrays containing 15,000 known or predicted genes of the *Drosophila melanogaster* genome were processed. These arrays were used to identify genes that were differentially expressed in *p38b* compared with *y w* male adult flies. Each sample contained a pool of 40 males, 2 to 4 d old. The statistical significance (false-discovery rate, q-value) and the ratio of the changes in expression were calculated using Significance Analysis of Microarray software following LOWESS normalization. The expression fold changes were presented as ratios.

Immune Response Assays. An overnight culture of *Enterobacter cloacae* or *Listeria monocytogenes* was suspended in PBS and injected into 2- to 4-d-old adult flies using a Hamilton G33 needle. *Beauveria bassiana* (Mycotrol) was cultured in a petridish with potato agar for 6 d at 25 °C. Anesthetized adult flies were rolled on the petridish with fungal spores. Flies were allowed to recover at 29 °C, and live flies were counted every 12 h or daily. About 60 2- to 4-d-old adult flies were used for each assay. Three independent assays were made for each genotype. One-hundred microliters of PAO1 or PAOR3 suspending culture ($O.D.^{600}$ = 1.0) was introduced into each fly vial with sterilized food and overnight deposited eggs for natural infection of *Pseudomonas aeruginosa*.

RT-PCR. Total RNA was isolated from adult flies or S2 cells using TRIzol Reagent (Invitrogen; catalog #15596–018). CDNA was synthesized with oligo-dT primers and SuperScript II Reverse Transcriptase RT-PCR System (Invitrogen) and analyzed by PCR with gene-specific primers. Quantitative RT-PCR was then done on the Applied Biosystems ABI Prism 7700 or 7900 Sequence Detection System using the SYBR Green PCR master mix (ABI catalog #4309155). All assays were done in triplicate and normalized to *rp49* levels, and errors were propagated in all calculations. Primer sequences are available on request.

RNA Interference. Double-stranded RNA (dsRNA) was produced using a MEGAscript RNAi Kit (Ambion). S2 cells were incubated with serum-free medium containing 25 μg/mL dsRNA for 30 min. The RNAi-treated cells were grown in serum-containing medium for 72 h before immune response experiments. The *E. cloacae*-induced immune response was examined using S2 cells, as described previously (2). Cells were collected 30 min after induction and were subjected to Western blot analysis with anti-phospho-p38 antibodies (Santa Cruz Biotechnology).

SDS/PAGE and Immunoblotting. Live *Drosophila* larvae, adults, or S2 cells were ground directly into 100 μL of SDS/PAGE sample buffer on ice and then boiled for 5 min. Samples (10 μL) were resolved by SDS/PAGE on 4 to 20% gradient Novex Tris-Glycine gels (Invitrogen). Native PAGE was used to detect heat-shock factor (Hsf) trimerization, as described previously (3). Gels were blotted and blots were processed by standard methods using 5% skim milk in Tris-buffered saline (TBS) that consists of 20 mM TrisCl (pH 7.5) and 154 mM NaCl with 0.1% Tween-20 for blocking and incubation steps. Probed blots were rinsed in TBS plus 0.1% Tween, developed with the luminescent substrate Super Signal West Pico (Pierce), and then exposed to film. Pri-

mary antibodies were diluted 1:1,000 to 1:10,000 and incubated overnight at 4 °C. For loading controls, monoclonal antibodies against α-tubulin (M1α; Sigma) were used. Affinity-purified, HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Pierce) were diluted 1:5,000 and incubated 1 h at 22 °C. Molecular mass standards (10–250 kDa) were Precision Plus All Blue prestained (Bio-Rad).

Light Microscopy and Imaging. All imaging was done on an Axioscop microscope with an Axiocam MRC color digital camera (Zeiss). Wandering third instar larvae were dissected with fine dissecting forceps in a drop of PBS. Dissected larval hindguts and fatbodies are fixed with 2% formaldehyde in PBS for 10 min and washed in PBS with 0.1% Triton-X100 (PBT). Next, 2% BSA in PBT was used as a blocking solution for 1 h before incubation with the primary antibody (1:10 monoclonal α-Dorsal; Iowa Hybridoma Bank) or Alexa Fluor 488 phalloidin (1:2,000; Molecular Probes) overnight at 4 °C. The labeling was visualized with fluorochrome-labeled secondary antibodies (Vector Laboratories). Labeled samples were washed in PBS and mounted using Vectorshield (Vector Laboratories) on glass slides.

Transmission Electron Microscopy. *Drosophila* larvae were dissected into Schneider medium, and the guts were immediately fixed in situ with 2.5% glutaraldehyde, 1% paraformaldehyde, 1% potassium ferrocyanide solution in 0.1 M cacodylate buffer, pH 7.4, for 80 min at room temperature. The samples were rinsed three times in a 0.1 M cacodylate buffer then postfixed with a 1% osmium tetroxide, 1% potassium ferrocyanide solution in 0.1 M cacodylate buffer for 1 h at room temperature. Dehydration of the guts was performed in an ascending series of ethanol concentrations, and then the samples were embedded in Epon 812. The guts were cut at 0.5 μm (semithin sections) for light microscopy or at 60 nm (ultra-thin sections) for transmission electron microscopy with a Leica ultramicrotome. Semithin sections were stained with methylen blue and Azur II and observed under an Axiophot Zeiss microscope. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and observed with a Philips CM100 electron microscope.

1. Craig CR, Fink JL, Yagi Y, Ip YT, Cagan RL (2004) A *Drosophila* p38 orthologue is required for environmental stress responses. *EMBO Rep* 5:1058–1063.
2. Park JM, et al. (2004) Targeting of TAK1 by the NF-kappa B protein Relish regulates the JNK-mediated immune response in *Drosophila*. *Genes Dev* 18:584–594.
3. Westwood JT, Clos J, Wu C (1991) Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. *Nature* 353:822–827.

Fig. S1. (*A* and *B*) Mutants of *p38a, p38b, and p38c*. Structural organization of *p38a* and *p38c* (*A*), *p38b* (*B*). The locations and orientations of the three *p38* genes are shown at the top with respect to the genomic maps below, in which the location of the *P*-element insertions are indicated. Numbers under the genomic maps indicate the locations on the chromosomes. The exons corresponding to the *p38a*, *CG6178*, and *p38b* transcripts indicate the ORF (filled in blue) and the untranslated regions (5′UTR and 3′UTR, white). The bars filled in yellow represent the ORFs of the genes predicted by the genome project. Arrows show the orientations of the genes or *P* elements. The deleted regions are indicated as red dotted lines at the bottom. (*C* and *D*) RT-PCR was performed to confirm presence of the *p38* mutants; *p38a*^{13}, *p38b*^{156A}, *p38c*^{7} appeared to be *p38a*, *p38b*, and *p38c* null alleles, respectively, but *p38a*^{1} is a double mutant for *p38a* and *p38c*.

Fig. S2. (*A*) Lifespan of *p38b;p38a* double mutants under sterile conditions. Lifespan was measured by using adult females and males tested in triplicate at 30 flies per vial at 25 °C. Flies were transferred every 4 d, and the number of dead flies was recorded. (*B*) Third instar larvae of *p38a*, *p38b*, *p38c*, and *p38a p38c* mutants. No melanization was observed.

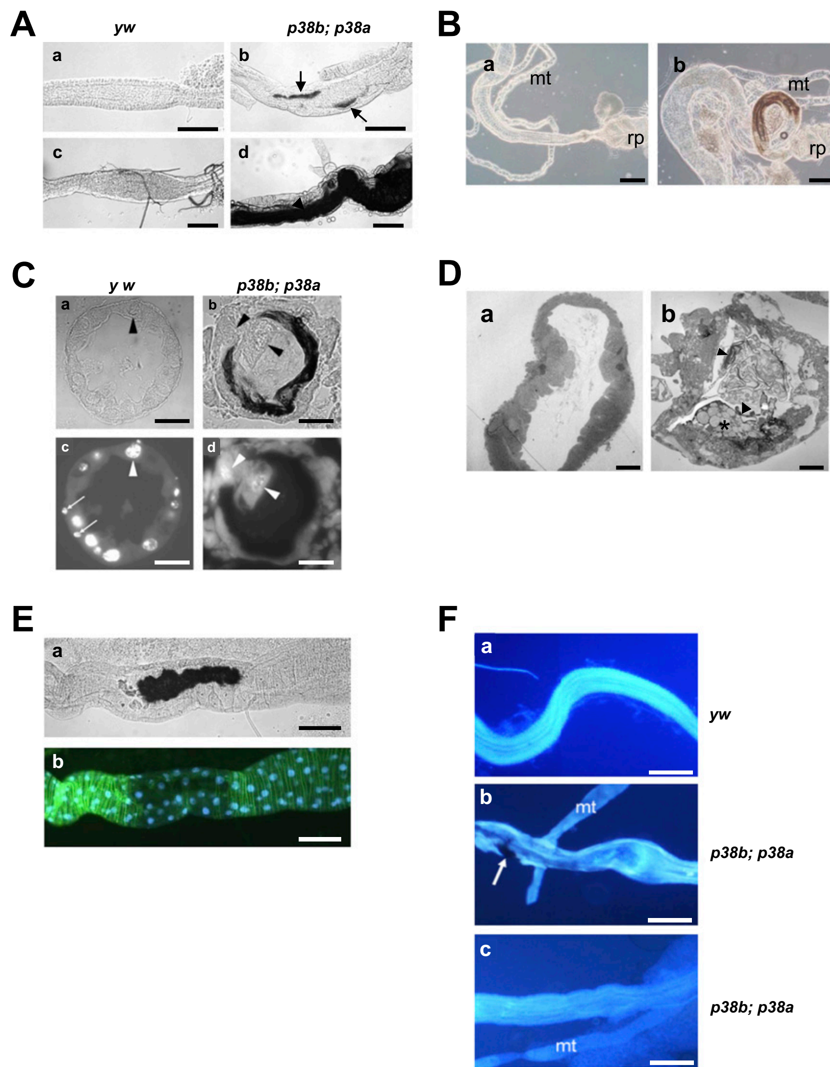

Fig. S3. (*A*) Hindgut defects of *p38b;p38a* double mutants. Nomarski images of hindguts that were dissected from late second instar larvae (a and b) and late third-instar larvae (c and d) of wild-type control (a and c) and the *p38b;p38a* double mutant (b and d). The arrows in panel b point to two separated sites with melanization. Note the expansion of melanization in late-stage hindgut from *p38b;p38a* double mutant (d). (Scale bars, 100 μm.) (*B*) Melanization was not observed in hindguts from 2-d-old *y w* adult (a) but in *p38b;p38a* double mutants (b). (Scale bars, 150 μm.) (*C*) Micrographs of hindgut cross sections. Nomarski images of wild-type control (a) and *p38b;p38a* double mutants (b). Fluorescent images (c and d) with DAPI staining to show nuclei, show the same view as in a and b, respectively. Hindguts of third instar larvae were removed, and paraffin embedding and sectioning were performed. Arrowheads point to nuclei of epithelial cells. The arrows in c point to nuclei of muscle cells that line the outer surface of epithelial cells. (Scale bars, 20 μm.) (*D*) Electron micrographs of hindgut cross sections from wild-type (a) and the *p38b;p38a* double mutants (b). The arrow and arrowhead point to the accumulated chitin masses and yeast cells in the lumen of the hindgut of *p38b;p38a*, respectively; the asterisk indicates the large vacuoles in the epithelial cells of the *p38b;p38a* hindgut. (Scale bars, 15 μm.) (*E*) Hindgut of *p38b;p38a* mutant was dissected out and stained with DAPI (DNA, blue) and Alexa-phalloidin (muscle fibers, green). Nomarski (a) and fluorescence (b) images were taken under a Zeiss Axiovision microscope. (Scale bars, 80 μm.) (*F*) Micrographs of hindgut stained with Calcofluor. Fluorescent images of hindguts from wild-type control (a) and *p38b;p38a* double mutants (b and c) were taken under the same condition. The midstage instar larvae were feed with 0.1% Calcofluor White Stain (Fluka) for 3 h and then hindguts were dissected. The arrow in b points to a melanotic mass in hindgut of *p38b;p38a* double mutant. Note the weakening of fluorescent intensity which represented thickness of chitin lining on the surface of gut lumen in *p38b;p38a* double mutant under standard culture condition (b) and sterile condition (c). (Scale bars, 150 μm.)

Fig. S4. (*A*–*D*) Susceptibility of *p38b;p38a, Tl* and *Rel* mutant flies to different pathogens. Compared with parental strain *y w*, *p38b;p38a* mutant flies are clearly more susceptible to three of the pathogens tested but not to *Escherichia coli. Rel* mutant flies are more susceptible to Gram-negative bacteria (*A* and *B*) and *Tl* mutants are more susceptible to Gram-positive bacteria and fungus (*C* and *D*). Groups of 60 male adults of each genotype, 2 to 4 d old, were pricked with concentrated *E. coli*, *P. aeruginosa, Staphylococcus aureus* (20 O.D.A600) or *Aspergillus fumigatus* spores (10^9 spores/mL).

Fig. S5. General statistics on the p38b-regulated genes. (*A*) A cluster image of the 309 p38-regulated genes. The expression profiles in flies of *p38b* and wild-type (*y w*) without or with septic injury in *E. cloacae* (Ec) or *L. monocytogenes* (Lm) are shown. Red and green indicate higher and lower mRNA levels respectively. (*B* and *C*) Graphs show the number of the genes differentially expressed in wild-type and *p38b* mutant flies responding to basal or septic injury with Ec and Lm.

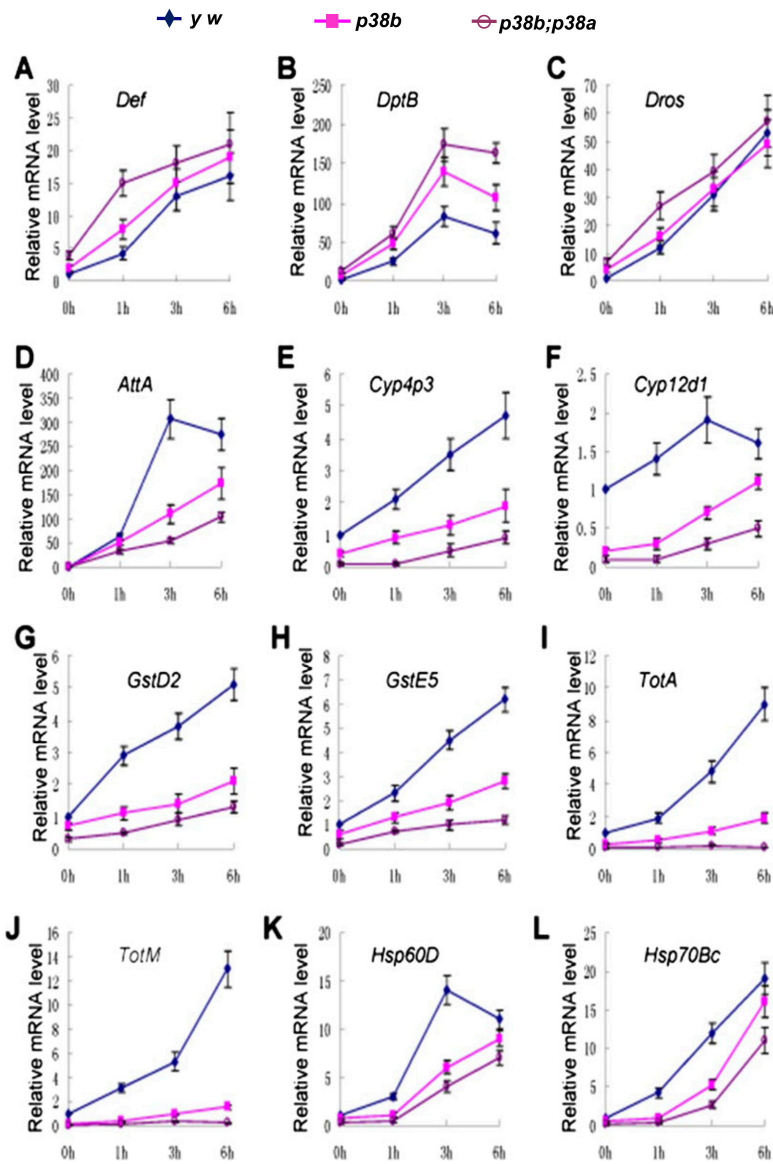

Fig. S6. The expression of selected p38 target genes characterized from microarray analysis before or after septic infection with a mixture of *E. cloacae* and *L. monocytogenes* was determined by quantitative RT-PCR normalized with internal control *rp49*. *Defensin* (*A*), *Diptericin B* (*B*), and *Drosomycin* (*C*) are up-regulated, and *Attacin A* (*D*), *Cyp4p3* (*E*), *Cyp12d1* (*F*), *GstD2* (*G*), *GstE5* (*H*), *TotA* (*I*), *TotM* (*J*), *Hsp60D* (*K*), and *hsp70Bc* (*L*) are down-regulated in *p38b* and *p38b;p38a* mutant flies. Data from three independent experiments are expressed as mean ± SEM.

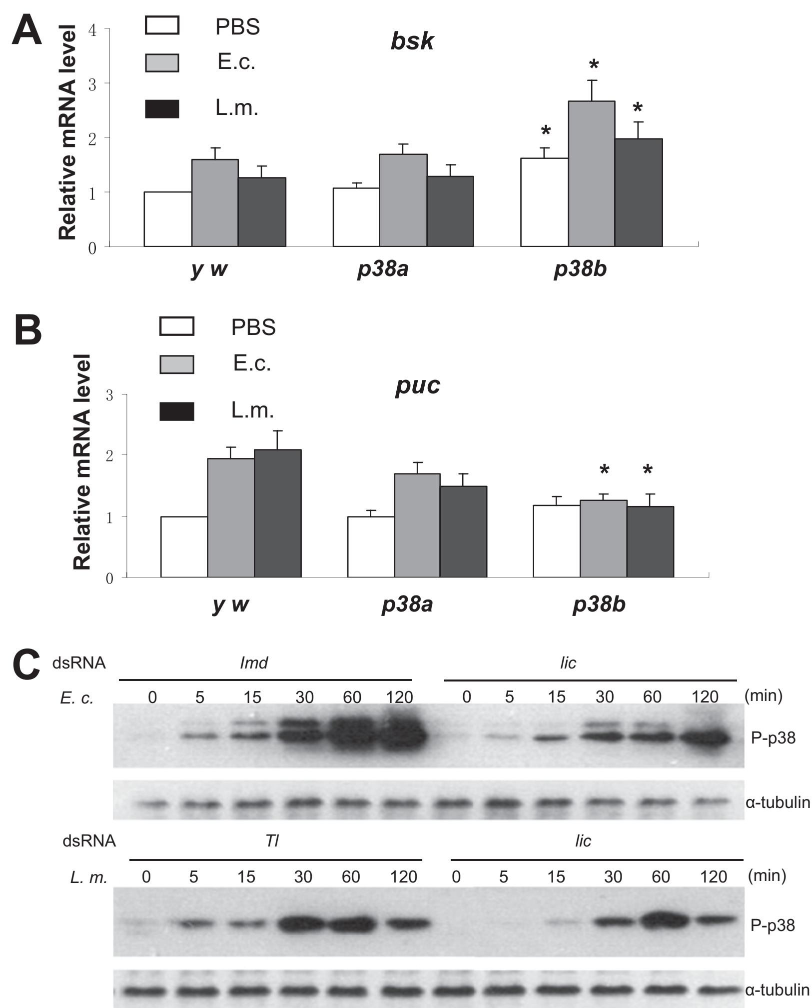

Fig. S7. (*A*) Relative mRNA levels of *bsk* in wild-type, *p38a*, and *p38b* mutants are shown with quantitative RT-PCR, normalized with the internal control *rp49*. Data are mean (± SEM) fold changes in *bsk* mRNA levels. **p38b* vs. *y.w*, $P < 0.02$. (*B*) Relative mRNA levels of *puc* in wild-type, *p38a*, and *p38b* mutants are shown with quantitative RT-PCR, normalized with the internal control *rp49*. Data are mean (± SEM) fold changes in *puc* mRNA levels. **p38b* vs. *y w*, $P < 0.01$. (*C*) Phophorylation of p38 was measured in S2 cells with RNAi knockdown of *Imd*, *Tl*, and *lic* at different time points after treatment with Gram-negative *E. cloacae* or Gram-positive *L. monocytogenes*. Phosphorylation was assayed by Western blotting using phospho-specific antibodies of p38. α-Tubulin is shown as a loading control.

Other Supporting Information Files

Dataset S1 (XLS)
Dataset S2 (XLS)