A Non-Redundant Role for Drosophila Mkk4 and Hemipterous/Mkk7 in TAK1-Mediated Activation of JNK

Peter Geuking1, Rajesh Narasimamurthy1,a, Bruno Lemaitre2,b, Konrad Basler1,*a, François Leulier2,nc,*

1 Institut für Molekularbiologie, Universität Zürich, Zürich, Switzerland, 2 Centre de Génétique Moléculaire, FRE 3144 CNRS, Centre de Recherche de Gif, Gif-sur-Yvette, France

Abstract

Background: The JNK pathway is a mitogen-activated protein (MAP) kinase pathway involved in the regulation of numerous physiological processes during development and in response to environmental stress. JNK activity is controlled by two MAPK kinases (MAPKK), Mkk4 and Mkk7. Mkk7 plays a prominent role upon Tumor Necrosis Factor (TNF) stimulation. Eiger, the unique TNF-superfamily ligand in Drosophila, potently activates JNK signaling through the activation of the MAPKKK Tak1.

Methodology/Principal Findings: In a dominant suppressor screen for new components of the Eiger/JNK-pathway in Drosophila, we have identified an allelic series of the Mkk4 gene. Our genetic and biochemical results demonstrate that Mkk4 is dispensable for normal development and host resistance to systemic bacterial infection but plays a non-redundant role as a MAPKK acting in parallel to Hemipterous/Mkk7 in dTAK1-mediated JNK activation upon Eiger and Imd pathway activation.

Conclusions/Significance: In contrast to mammals, it seems that in Drosophila both MAPKks, Hep/Mkk7 and Mkk4, are required to induce JNK upon TNF or pro-inflammatory stimulation.

Introduction

The JNK pathway, one of the three major classes of mitogen-activated protein (MAP) kinase pathways (Erk, p38 and JNK), is induced by pro-inflammatory cytokines, such as Tumor Necrosis Factor (TNF) and Interleukin-1 (IL-1), and several forms of environmental stress (e.g. osmotic stress, irradiation, and oxidative stress) [1]. In mammals, JNK is reported to be activated by two MAPK kinases (MAPKK) Mkk4 and Mkk7, with Mkk7 as the major MAPKK in TNF- or IL-1-induced JNK activation while both, Mkk4 and Mkk7, are required for stress induced activation of JNK [2]. In mammals, Mkk7 is a specific activator of JNKs while Mkk4 can also phosphorylate p38 MAPKs [3]. In mice, analysis of the relative contribution of Mkk4 and Mkk7 to JNK activation has been complicated by the fact that Mkk4 and Mkk7 single mutants are embryonic lethal [2].

Drosophila orthologs of Mkk4 and Mkk7 have been identified [4,5,6]. So far, only mutations in hemipterous/Mkk7 (hep), have been isolated [4]. Hep phosphorylates and activates the Drosophila JNK, Basket [Bsk] [7] and null mutations in hep lead to a defect in dorsal closure, a well characterized process in the Drosophila embryo that entirely depends on JNK signaling [4,7]. In contrast to mammals, Drosophila Mkk4 only activates JNK but not p38 in vitro [5,8], however this remains controversial [6]. To date no mutants for Drosophila Mkk4 have been identified and its functional relevance towards JNK activation therefore remains elusive. Based on the embryonic lethality of hep mutants it is obvious that Mkk4, which is expressed during embryonic development, cannot substitute for Hep function in this process. Although it has been reported that in mammals Mkk4 and Mkk7 may synergistically activate JNK [9], it does not seem to be the case for Hep-mediated Bsk activation during dorsal closure.

In a dominant suppressor screen for new components of the Eiger-JNK-pathway in Drosophila [10], we have identified an allelic series of the Drosophila Mkk4 gene. Our genetic and biochemical experiments now demonstrate a non-redundant role for Mkk4 as a MAPKK acting in parallel of Hep/Mkk7 in dTAK1-mediated JNK activation upon Eiger and Imd pathway activation.

Results and Discussion

Mutations in Mkk4 Suppress Eiger Mediated Small Eye Phenotype

In a dominant suppressor screen for new components of the Eiger-JNK-pathway in Drosophila [10], we identified 21 EMS mutations suppressing Eiger-induced cell death in the eye that
mapped genetically very close to a deficiency (Df(3R)Exel6149) that also suppresses the Eiger-induced small eye phenotype (Figure 1D). This deficiency removes 26 genes including Mkk4. We sequenced the coding region of Mkk4 in those EMS alleles and molecular lesions were detected in all of them. Several mutations create a premature stop codon in the open reading frame and therefore

Figure 1. Mkk4 triggers Eiger-mediated small eye phenotype. (A) Schematic representation of Mkk4. Alleles (black) and Ser/Thr phosphorylation sites (green) are indicated. (B)–(J) are in a GMR-egr (GMR-Gal4, UAS-egr) background. (B) GMR-Gal4/+ control eye. (C) GMR-egr/+ small eye. (D) GMR-egr/+/Df(3L)Exel6149/+. (E) GMR-egr/++; Mkk4G680/+. Removing one copy of Mkk4 suppresses the small eye phenotype. (F) GMR-egr/++; Mkk4G680/Df(3R)Exel6149. Removing both copies of Mkk4 does not improve the suppression. (G) hep1/GMR-egr/+. Males hemizygous mutant for a hypomorphic hep allele display a strong suppression of the small eye. (H) GMR-egr/tub-Mkk4; Mkk4G680/+. A Mkk4 rescue transgene reverts the dominant suppression observed by loss of one copy of Mkk4. (I) GMR-egr/UAS-Mkk4. Co-expression of Mkk4 has dominant negative effect on Eiger signal transduction. (J) GMR-egr/UAS-Mkk4Asp. The same effect is observed for Mkk4Asp. (K)–(O) are in a GMR-hepCA (GMR-Gal4, UAS-hepCA) background. (K) GMR-hepCA/+ (25°C). (L) GMR-hepCA/UAS-Mkk4 (25°C). Co-expression of Mkk4 also suppresses the small eye phenotype induced by HepCA. (M) GMR-hepCA/UAS-Mkk4Asp (25°C). Co-expression of Mkk4Asp does not suppress the small eye phenotype induced by HepCA. (N) GMR-hepCA/+ (18°C). Weaker expression of hepCA leads to a less severe small eye phenotype. (O) GMR-hepCA/UAS-Mkk4G587/Mkk4G673 (18°C). This phenotype is not suppressed, even when both copies of Mkk4 are removed. (P) Alignment of the amino-acid sequence of Hep and Mkk4 catalytic region. The mutations introduced in HepCA, Mkk4Asp and Mkk4Mut are indicated in red. doi:10.1371/journal.pone.0007709.g001

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likely represent null alleles (Table 1, Figure 1A). Of note, all the 21 alleles behaved the same and lead to a strong suppression of the Eiger-induced small eye phenotype.

Mkk4 mutant flies are viable and do not show obvious morphological defects over Df(3R)Exel5149 or in heteroallelic combinations. In some cases homozygous lethality is observed which is most likely due to second mutations on the chromosome.

Removing a single copy of Mkk4 leads to a potent suppression of the Eiger-induced small eye phenotype (Figure 1B–E). Removing two copies of Mkk4 does not significantly enhance this suppression (Figure 1F). Therefore, in this context Mkk4 mutations are dominant suggesting that Mkk4 is haplo-insufficient for Eiger-induced small eye phenotype. Introducing a tubulin-Mkk4 rescue transgene reverts the observed dominant suppression indicating that indeed Mkk4 is responsible for this effect (Figure 1H). It is important to note that hemizygous males for the hypomorphic hep allele also show a very good suppression of the Eiger-induced small eye phenotype [11] (Figure 1G), indicating that in Drosophila both MAPKKs, Mkk4 and Hep/Mkk7, are rate limiting for proper transduction of the Eiger signal. This demonstrates that in Drosophila, in contrast to mammals, Mkk4 is haplo-insufficient for TNF superfamily ligand (Eiger)-mediated JNK activation.

**Mkk4 Acts as MAPKK for dTAK1 Mediated Activation of JNK**

To confirm that Mkk4 indeed acts, like Hep, at the level of a MAPKK in the JNK pathway, we performed epistasis experiments in flies and cells as well as protein interaction studies. Removing one (not shown) or both copies of Mkk4 does not suppress the small eye phenotype induced by expression of an activated version of hep (hep<sup>CA</sup>) in the Drosophila eye [12] (Figure 1N and O). This result suggests that Mkk4 does not genetically function downstream of Hep. In S2 cells, the expression of the MAPKK K dTAK1 potently activates the JNK pathway, which leads to the activation of the API-luciferase-reporter gene (Figure 2A). Co-RNAi against hep and Mkk4 reduces this activity (Figure 2A). However single RNAi treatment against either of the two kinases was not sufficient to reduce the luciferase signal (Figure 2A). In S2 cells the JNK pathway is also activated in a dTAK1 dependent manner upon treatment by commercial preparation of LPS (Figure 3A) [13,14,15]. RNAi against either hep or Mkk4 reduces JNK activation upon commercial LPS treatment (Figure 3A) suggesting that both kinases are rate limiting in this situation. In agreement with this, the reduction in phosphorylated JNK levels is enhanced when both kinases are targeted by RNAi at the same time (Figure 3A). This last result confirms previous reports indicating that both, Mkk4 and Hep, are required to mediate JNK activation upon commercial LPS treatment [13,14]. Taking together our RNAi experiments in S2 cells place Mkk4 downstream of the MAPKK dTAK1 in the control of JNK, confirming that Mkk4 functions as a classical MAPKK. Further evidence suggesting that Mkk4 indeed acts as a MAPKK was obtained from protein interaction studies. When expressed in S2 cells, N-terminally HA tagged Mkk4 co-immunoprecipitated both, C-terminally FLAG tagged dTAK1 and Bsk. Indeed in CoIP experiments removing one or both copies of Mkk4 (Figure 2B) or in fly eyes (data not shown). Interestingly, wild type Mkk4 has a dominant negative effect when co-expressed with Eiger (Figure 1I) or Hep<sup>CA</sup> (Figure 1I) in flies. This may stem from its ability to interact with Bsk and dTAK1. Overexpressing Mkk4 may therefore titrate away Bsk and dTAK1. In an attempt to generate a constitutive active Mkk4 (Mkk4<sup>Asp</sup>), we introduced the Ser<sup>277</sup>→Asp and Thr<sup>281</sup>→Asp mutations, which corresponds to the mutations that were introduced to generate Hep<sup>CA</sup> [12] (Figure 1P). Surprisingly, Mkk4<sup>Asp</sup> is not constitutively active, neither in flies (not shown) nor in S2 cells (Figure 2B). However, expressing Mkk4<sup>Asp</sup> suppresses GMR-egr [Figure J] but not GMR- hep<sup>CA</sup> (Figure 1M). Finally a kinase dead version of Mkk4 (Mkk4<sup>WT</sup>) where mutations Ser<sup>277</sup>→Ala and Thr<sup>281</sup>→Val were introduced (Figure 1P) behaved identically to Mkk4<sup>WT</sup> (not shown) suggesting that the kinase activity of Mkk4 is not associated with its dominant negative effect upon overexpression. This effect may rather relate to differential binding ability towards dTAK1 and Bsk of Mkk4<sup>WT</sup> and Mkk4<sup>Asp</sup>. Indeed in CoIP experiments Mkk4<sup>Asp</sup> is still able to bind dTAK1 but no longer Bsk (Figure 2D). Altogether our results therefore demonstrate that Mkk4 is a MAPKK acting in parallel of Hep/Mkk7 and downstream of dTAK1 in the activation of Bsk/JNK upon both Eiger expression and Imd pathway activation by commercial LPS.

In contrast to the intrinsic activity of Hep<sup>CA</sup> (strong) and Hep<sup>WT</sup> (weak) (Figure 1,K and 2B) [12], wild type Mkk4 does not activate the JNK pathway when overexpressed in S2 cells (Figure 2B) or in fly eyes (data not shown). Interestingly, wild type Mkk4 has a dominant negative effect when co-expressed with Eiger (Figure 1I) or Hep<sup>CA</sup> (Figure 1I) in flies. This may stem from its ability to interact with Bsk and dTAK1. Overexpressing Mkk4 may therefore titrate away Bsk and dTAK1. In an attempt to generate a constitutive active Mkk4 (Mkk4<sup>Asp</sup>), we introduced the Ser<sup>277</sup>→Asp and Thr<sup>281</sup>→Asp mutations, which corresponds to the mutations that were introduced to generate Hep<sup>CA</sup> [12] (Figure 1P). Surprisingly, Mkk4<sup>Asp</sup> is not constitutively active, neither in flies (not shown) nor in S2 cells (Figure 2B). However, expressing Mkk4<sup>Asp</sup> suppresses GMR-egr [Figure J] but not GMR- hep<sup>CA</sup> (Figure 1M). Finally a kinase dead version of Mkk4 (Mkk4<sup>WT</sup>) where mutations Ser<sup>277</sup>→Ala and Thr<sup>281</sup>→Val were introduced (Figure 1P) behaved identically to Mkk4<sup>WT</sup> (not shown) suggesting that the kinase activity of Mkk4 is not associated with its dominant negative effect upon overexpression. This effect may rather relate to differential binding ability towards dTAK1 and Bsk of Mkk4<sup>WT</sup> and Mkk4<sup>Asp</sup>. Indeed in CoIP experiments Mkk4<sup>Asp</sup> is still able to bind dTAK1 but no longer Bsk (Figure 2D). Altogether our results therefore demonstrate that Mkk4 is a MAPKK acting in parallel of Hep/Mkk7 and downstream of dTAK1 in the activation of Bsk/JNK upon both Eiger expression and Imd pathway activation by commercial LPS.

Mkk4<sup>Asp</sup> is Dispensable for the Activation of the IKK/Relish Cascade by dTAK1

dTAK1 is an important MAPKKK regulating the activity of both the JNK and IKK/Relish branch of the Imd cascade, a signaling pathway regulating the expression of several immune effectors upon infection [15,16]. In absence of a functional Imd/IKK/Relish cascade as in dTAK1 mutants, flies are extremely sensitive to systemic infection by Gram-negative bacteria, including *Escherichia coli*. The resistance of Mkk4<sup>Asp</sup> mutant flies to systemic infection by *Erwinia* carotovora is phenotypically identical to flies expressing Mkk4<sup>WT</sup> (not shown). It is therefore likely that Mkk4 plays a role in the regulation of a systemic immune response in flies. However, this role is not essential for mediating the JNK response to Eiger expression in the eye.

**Table 1. Mkk4 allelic series.**

| Mkk4 allele | Lesion on DNA level (wt – mut) | Lesion on protein level |
|-------------|-------------------------------|------------------------|
| G48         | GCA → GTA                     | Ala101Lys              |
| G356        | 653bp insertion at Ser38      | AA11 of insertion is a STOP |
| G673        | CAG → TAG; TTC → TAC          | Gln665STOP; Phe184Tyr   |
| G344        | CGA → TGA                     | Arg154STOP             |
| G993        | GAT → AAT                     | Asp168Asn              |
| G451        | GTG → ATG                     | Val171Met              |
| G201        | GAT → AAT                     | Asp249Asn              |
| G341        | GTG → ATG                     | Val250Met              |
| G894        | GTG → GAG                     | Val250Asn              |
| G136        | CGG → TCG                     | Pro225Ser              |
| G863        | GGT → GAT                     | Gly269Asp              |
| G414        | CAG → TAG                     | Gln273STOP             |
| G39         | CGG → CTG                     | Pro292Leu              |
| G583        | CGG → TCG                     | Pro292Ser              |
| G657        | GAT → AAT                     | Asp308Asn              |
| G504        | GAG → AAG                     | Glu318Lys              |
| G270        | CCC → CTC                     | Pro325Leu              |
| G587        | TGG → TAG                     | Trp329STOP             |
| G1010       | 5′ splice site intron 3: AGGTT | -                       |
| G680        | CAA → TAA                     | Gln341STOP             |
| G262        | GTG → AGT                     | Val361Met              |

Molecular lesions identified in Mkk4. Alleles are ordered according to their position in the protein.
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Therefore, we investigated if Mkk4 is implicated in the control of the IKK/Relish branch of the Imd pathway. To this end we tested if an Mkk4 deficiency leads to similar immune phenotypes like dTAK1 loss of function. We challenged Mkk4 mutants with Ecc15 and monitored their survival over time. Figure 3B shows that in contrast to Relish and dTAK1 mutants, Mkk4 mutants survive like wild-type flies to this challenge. This result therefore suggests that Mkk4 is dispensable for the activation of the Imd/IKK/Relish cascade by dTAK1. Therefore, the involvement of Mkk4 in the Imd cascade is restricted to the dTAK1-mediated activation of the JNK branch (Figure 3A).

The Egr/dTAK1/Mkk4 Cascade Is Dispensable to Fight Gram-Positive Cocci Infections

Recently, Schneider and colleagues showed that eiger mutants are sensitive to systemic infection by gram-positive cocci, a type of extracellular bacteria [17]. In order to test if this egr related process relies on the same signaling cascade as the one activated in the eye

Figure 2. Mkk4 function as a MAPKK between dTAK1 and JNK/Bsk. (A) RNAi against Mkk4 and hep together significantly reduces dTAK1-induced AP-1-luciferase reporter activity. (B) In contrast to Hep and HepCA, Mkk4 and Mkk4Asp do not induce AP-1-luciferase reporter activity on their own. (C) Mkk4 physically interacts with dTAK1 and Bsk. (D) Mkk4Asp still interacts with dTAK1 but no longer binds to Bsk.

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Figure 3. Mkk4 activates the JNK branch of the Imd pathway, not the IKK/Relish branch. (A) RNAi against Mkk4 and/or hep reduces phosphorylated JNK levels induced by commercial LPS in S2 cells. (B) Survival analysis of w^1118 (closed diamond), Mkk4^G673/Df(3R)Exel6149 (closed square), dTAK1^11 (cross) and Relish^E20 (closed triangle) flies upon Erwinia carotovora carotovora 15 (Ecc15) septic injury.

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upon egr expression, we challenged Mkk4 mutants with Staphylococcus aureus, a gram-positive coccus (Figure 4A). We compared their viability to egr mutants and other mutants affecting the Toll signaling pathway, dPZ and PGRP-SA, which contribute to the resistance to systemic gram-positive cocci infection [18,19]. Figure 4A shows that although egr1 mutant show a slight increased susceptibility to this challenge compared to wild-type flies, however this was not as pronounced as dPZ and PGRP-SA mutants. Importantly, Mkk4 mutants behave like wild-type animals in this setting. These results corroborate our data showing that mutants behave like wild-type animals in this setting. These results mediate the not sensitive to Gram-positive cocci infection while being essential to Staphylococcus aureus (egr3 and egr66 alleles) mild susceptibility compared to wild-type flies and tested related or independent positive cocci infection. In order to confirm this phenotype, we type control line of the parental line initially used to generate Regg1; to create an isogenic wild-type control line of the Regg1 P-element insertion, Regg11c, and as wild-type flies to REGG1 null alleles are loss of function. eiger66 and egr1, and egr3 mutants to Gram-positive cocci is rather due to the genetic background of the eiger3 mutant lines (Figure 4B). The reduced pathogenicity of S.aureus compared to S.aureus revealed that the Regg1 and Regg11c fly lines show a mild susceptibility to this bacterial infection similarly to egr1 and egr66 mutants. Taken together, these results therefore suggest that the observed susceptibility of egr1 and egr3 mutants to Gram-positive cocci is rather due to the genetic background of the Regg1 line but not associated with egr loss of function.

Conclusion

In this study we have isolated for the first time an allelic series of Drosophila Mkk4. Using these mutants we showed that Mkk4 is dispensable for normal development and for host resistance to systemic bacterial infection. Our genetic and biochemical experiments demonstrate a non-redundant role for Mkk4 as a MAPKK acting in parallel to Hep/Mkk7 in dTAK1-mediated JNK activation in the Eiger and Imd pathways (Figure 5). In contrast to mammals, it seems that in Drosophila both MAPKs, Hep/Mkk7 and Mkk4, are required to induce JNK upon TNF or pro-inflammatory stimulation.

Methods

Fly Stocks

Fly stocks were maintained on polenta-agar medium at 23°C. w1118 and yw fly strains were used as controls when appropriate.

Figure 4. The Egr/dTAK1/Mkk4 cascade is dispensable to fight gram-positive cocci infection. (A) Survival analysis of yw (closed diamond), w1118 (closed square), Mkk4Δ/Δ(Df(3R)Exel6149) (cross), eiger1 (egr; closed triangle), Spz-72 (closed circle) and PGRP-SA<sup>seml</sup> (dash) flies upon Staphylococcus aureus (S.aureus) septic injury. (B) Quantitative RT-qPCR analysis of basal egr expression in yw, Regg1, Regg1<sup>1C</sup>, egr1, egr4 and egr66 mutant lines (Figure 4B). Upon S.aureus infection egr1 and egr4 flies behave similarly and show a mild susceptibility compared to wild-type flies and PGRP-SA<sup>seml</sup> mutant flies. However, the egr66 mutants which lack the entire egr coding region behave like wild-type controls (Figure 4C). When we repeated this experiment using another, less pathogenic, gram-positive coccus, Enterococcus faecalis, we observed the same pattern of results with egr1, egr4 and egr66 alleles, with egr66 being as susceptible as wild-type flies to E.faecalis (Figure 4D). The reduced pathogenicity of E.faecalis compared to S.aureus revealed that the Regg1 and Regg1<sup>1C</sup> fly lines show a mild susceptibility to this bacterial infection similarly to egr1 and egr66 mutants. Taken together, these results therefore suggest that the observed susceptibility of egr1 and egr3 mutants to Gram-positive cocci is rather due to the genetic background of the Regg1 line but not associated with egr loss of function.

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Stocks carrying the UAS-Mkk4 (Mkk4) and tubulinα1-Mkk4 transgene were obtained by standard P-element-induced transformation. dTAK1 and RelishE20, spzrm7, PGRP-SAseml, Regg1, egr1, egr3, egr66 and hep1 fly strains were described previously [4,11,20,21]. Regg1 was generated by classical transposase-mediated precise excision of the Regg1 P-element. Df(3R)Exel6149 was obtained from Bloomington stock center. UAS-hep, GMR-gal4 and UAS-egr were previously described [10,12].

Genetic Mapping of the Alleles

From our dominant suppressor screen no lethal complementation group on the third chromosome could be identified [10]. Based on this observation we decided to combine two strategies in order to map dominant suppressor mutations on the third chromosome. First, we screened the whole Exelixis deficiency kit [22] for dominant suppressors of the Egr-induced small eye phenotype. Df(3R)Exel6149 was selected as a dominant suppressor deficiency. This deficiency removes 26 genes including Mkk4 and maps to the cytological location 85A [22]. Second, we performed a classic genetic mapping by meiotic recombination. In absence of a homozygous phenotype we made use of the dominant suppressor phenotype in our sensitized background. To this end, three RFP (red fluorescent protein)-marked insertions at positions 62B, 85E and 92A were used as genetic markers (one of them located close to Df(3R)Exel6149 at position 85A, which was isolated as a dominant suppressor from the deficiency screen). Chromosomes carrying a suppressor mutation were allowed to recombine with the RFP-marked chromosome in females. These virgins were crossed back to GMR-egr/CyO males. The number of non-CyO RFP(+) progeny (sorted under a fluorescent binocular) with a suppressed eye phenotype in relation to the number of non-CyO RFP(+) progeny with a small eye reflects the relative genetic distance to the RFP insertion. In stocks carrying a suppressor mutation that mapped very close to the RFP(+) insertion at 85E (only 1-3% recombination frequency between suppressor mutation and RFP(+) at 85E), the Mkk4 gene was checked for point mutations by sequencing. The Mkk4 gene was chosen because it was the most evident candidate from the 26 genes deleted in Df(3R)Exel6149.

Transgenes

The Mkk4 full-length cDNA (RE70055) was cloned into pUAST [23] and into a vector containing the tubulinα1 promoter [24], respectively. For UAS-Mkk4Δp and UAS-Mkk4Δmr the corresponding mutations were introduced by classical site directed mutagenesis.
Sequencing
Genomic DNA was amplified by PCR using evenly spaced primers in the Mkk4 coding region. PCR products were analyzed by standard sequencing.

Drosophila Cell Culture and Transfection
Schneider (S2) cells were cultured in Schneider’s Drosophila medium (Invitrogen, San Diego) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 25°C. Cells were transfected with expression vectors, using Cellfectin (Invitrogen) according to the manufacturer’s protocol. Expression vectors: pUAST-Mkk4, pUAST-HA-Mkk4, pUAST-Mkk4Δ, pUAST-HA-Mkk4Δ, pUAST-dTAK1, pUAST-dTAK1-FLAG, pUAST-hepC, pUAST-hepCΔ, pUAST-bock-FLAG, prb-Gal4, pUAST-GFP.

Immunoprecipitation and Immunoblotting
S2 cells (0.75x10^5 cells/well) were seeded into a 12-well plate. One day after seeding cells were transfected with the indicated expression vectors. Forty-eight hours after transfection the cells were harvested and lysed in lysis buffer containing 150 mm NaCl, 50 mm Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.5% deoxycholic acid, and protease inhibitors (Complete Mini; Roche, Indianapolis). Lysates were mixed with an anti-HA antibody and 25 μl of Protein-A sepharose beads and allowed to rotate at 4°C overnight. The beads were then collected and washed with the lysis buffer four times. Proteins were eluted from the beads and resolved on a 4–12% NUPAGE gel system (Invitrogen) and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with anti-FLAG M2 antibody (Sigma) followed by appropriate secondary antibodies conjugated with horseradish peroxidase (HRP). Signals were detected with ECL reagents (Amersham, Arlington Heights, IL).

LPS Treatment
S2 cells were treated with dsRNA (15 μg/10^6 cells) and split into two halfs. One half was left untreated and the other half was treated with lipopolysaccharide (LPS) (Sigma) at a concentration of 50 μg/ml for 10 min (note that commercial preparation of LPS contains peptidoglycan, which potently induces the IMD signaling cascade [21]). The cells were then lysed in lysis buffer. The lysates were analyzed by immunoblotting to detect phosphorylated JNK of a pool of 15 males.

Luciferase Assay
S2 cells (0.4x10^6 cells/well) were seeded into a 24-well plate. One day after seeding cells were transfected with an AP1-luciferase reporter plasmid along with the indicated expression vector. The total DNA concentration (1 μg) was kept constant by supplementing with empty vector. Forty-eight hours after transfection, cells were harvested, lysed in passive lysis buffer, and luciferase activity was measured using the dual luciferase assay system (Promega). The values shown reflect the relative luciferase activity: the ratio of firefly (AP1 luciferase) and tub-revilla luciferase activity of one representative experiment in which each transfection was made in duplicate.

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Double-Stranded RNA Production
Double-stranded RNA (dsRNA) was prepared as described by the Dixon lab [25]. Briefly, using PCR products as templates, the MEGASCRIPT T7 transcription kit (Ambion, Austin, TX) was used to produce RNA according to the manufacturer’s protocol. RNA products were ethanol precipitated and resuspended in DEPC-treated water. dsRNA was generated by annealing at 65°C for 30 min followed by slow cooling to room temperature. The following sets of forward and reverse primers were used (17 sequences are not indicated):

- **Mkk4:** sense 5’-caatatcccccggtcagtaaq-3’, antisense 5’-caatctcc-gattgataaatcagt-3’
- **hep:** sense 5’-gcaatctcagtaatcagtgc-3’, antisense 5’-ttgagcttg-gateccatagg-3’
- **Bsk:** sense 5’-gcggtcaaggaagccgtc-3’, antisense 5’-tcagcatcac-acac-3’
- **dTAK1:** sense 5’-gtgacacatcaggtggc-3’, antisense 5’-gggcatggg-agttccagc-3’
- **GFP:** sense 5’-gaattctttctaggatggcc-3’, antisense 5’-gccacttgttaacctcaggc-3’

Quantitative Real-Time PCR
SYBR Green quantitative real-time PCR analysis was performed as previously described [21]. Primer pairs for eg (sense: 5’- TAATCTCAGCCAGTT-3’, antisense 5’-ATGTTGCTC-CGCCACAAC-3’), and RpL32 (sense, 5’-GAC GCT TCA AGG GAC AGT ATC TG-3, and antisense, 5’-AAA CGC GGT TCT GCA TGA G-3’) were used to detect target gene transcripts. The amount of eg mRNA detected was normalized to control RpL32 mRNA values. Normalized data was used to quantify the relative levels of a given mRNA according to cycling threshold analysis (ΔCt). Relative ΔCt referred to ΔCt of wild-type controls were anchored in 1 to indicate fold-induction. Graphs represent the mean and S.D of relative ratios detected in 3 biological repetition of a pool of 15 males.

Bacterial Strains and Infection Experiments
Systemic infections were performed by prickling 60 adult males of 4 to 7 days old in the lateral thoracic region with a thin needle previously dipped into a concentrated pellet of the following bacteria. *Escherichia coli* (OD 170, 29°C); *Enterococcus faecalis* (OD 10, 25°C) or *S. aureus* (OD 10, 25°C). Flies were incubated at the indicated temperatures and their survival was monitored twice every day.

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Author Contributions
Conceived and designed the experiments: PG BL KB FL. Performed the experiments: PG RN FL. Analyzed the data: PG RN BL KB FL. Wrote the paper: PG FL.
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