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# Cooperative control of *Drosophila* immune responses by the JNK and NF-κB signaling pathways

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**Jun N-terminal kinase (JNK) signaling is a highly conserved pathway that controls both cytoskeletal remodeling and transcriptional regulation in response to a wide variety of signals. Despite the importance of JNK in the mammalian immune response, and various suggestions of its importance in *Drosophila* immunity, the actual contribution of JNK signaling in the *Drosophila* immune response has been unclear. *Drosophila* TAK1 has been implicated in the NF-κB/Relish-mediated activation of antimicrobial peptide genes. However, we demonstrate that Relish activation is intact in *dTAK1* mutant animals, and that the immune response in these mutant animals was rescued by overexpression of a downstream JNKK. The expression of a JNK inhibitor and induction of JNK loss-of-function clones in immune responsive tissue revealed a general requirement for JNK signaling in the expression of antimicrobial peptides. Our data indicate that dTAK1 is not required for Relish activation, but instead is required in JNK signaling for antimicrobial peptide gene expression.**

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## Introduction

Innate immune responses are critical for a rapid host defense against pathogens. The signaling pathways that control these responses are present in all multicellular organisms, ranging from humans to flies, and are remarkably well conserved. Although the innate response lacks the antigen recognition capacity of vertebrate adaptive immunity, it is nevertheless complex and crucial for host survival (Medzhitov and Janeway, 1998; Dabbagh and Lewis, 2003; Takeda *et al*, 2003; Vercelli, 2003). *Drosophila melanogaster* is a proven genetic model organism for the study of innate immunity and has provided invaluable insights into the control of responses to infection.

Toll and Imd are the founding members of two principal innate immune response signaling pathways in *Drosophila*. Toll signals through two NF-κB/Rel family transcription factors, Dif and Dorsal, and is required for responses to fungal and Gram$^+$ bacterial infections (Rutschmann *et al*, 2000a; De Gregorio *et al*, 2002). Imd signaling controls primarily Gram$^-$ bacteria-specific responses through the cleavage and activation of a third Rel family transcription factor, Relish, by the *Drosophila* caspase Dredd (Stöven *et al*, 2000, 2003). Relish activation also requires an IκB kinase (IKK) complex that is itself activated by Imd signaling (Rutschmann *et al*, 2000b; Silverman *et al*, 2000; Lu *et al*, 2001; Stöven *et al*, 2003). The transcriptional targets of Dif and Relish are not entirely distinct. For example, *cecropinA* expression requires either Relish or Dif, or both, depending on the type and strain of infecting microorganism (Hedengren-Olcott *et al*, 2004). More than 20 *Drosophila* genes have been implicated in these signaling pathways and nearly all of them have mammalian homologues with conserved immune functions (Brennan and Anderson, 2004).

Jun N-terminal kinase (JNK) signaling has been linked to stress responses, cell migration, apoptosis, and immune responses in both insects and mammals (Sluss *et al*, 1996; Leppèa and Bohmann, 1999; Stronach and Perrimon, 1999; Boutros *et al*, 2002; Dong *et al*, 2002). JNK activity can be induced by infection, lipopolysaccharide, and inflammatory cytokines such as tumor necrosis factor (TNF) in flies and mammals (Sluss *et al*, 1996; Boutros *et al*, 2002; Dong *et al*, 2002; Igaki *et al*, 2002; Moreno *et al*, 2002). Null mutations in JNK signaling components are typically embryonic lethal in flies and thus unlikely to appear as targets of mutagenesis screens designed to detect immune response genes in living animals. An exception to this rule is dTAK1. Overexpression and dominant-negative studies indicated that dTAK1 can act as a JNK kinase kinase (Mihaly *et al*, 2001; Igaki *et al*, 2002; Moreno *et al*, 2002).

Previously characterized dTAK1 mutations, however, showed no apparent JNK-like phenotype, but failed to express Relish-dependent antimicrobial peptides, suggesting a role in the Imd pathway (Vidal *et al*, 2001). Previous epistasis analysis using the UAS/GAL4 overexpression system (Brand and Perrimon, 1993) to ectopically express *dTAK1* placed *dTAK1* downstream of *imd* and upstream of the *IKK* complex in the Relish signaling pathway (Vidal *et al*, 2001). *In vitro* experiments implicated *dTAK1* in the IKK-dependent phosphorylation of Relish in S2 cells (Silverman *et al*, 2003).

We uncovered evidence for a Relish-independent function of dTAK1 in the control of antimicrobial peptide gene

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expression. Several aspects of Relish activation appeared normal in infected *dTAK1* mutant animals, including cleavage, nuclear localization, and promoter binding. We therefore tested if JNK pathway components mediated dTAK1 function in the immune response. We report here several lines of evidence for dTAK1 acting through the JNK cascade in the innate immune response. First, overexpression of Hemipterous, a JNKK, rescued *attacin* and *diptericin* expression in dTAK1 mutant animals, whereas overexpression of the downstream Imd component Dredd did not. Second, we found that expression of the Puckered (Puc) phosphatase, an inhibitor of JNK activity, suppressed the expression of antimicrobial peptide genes. To directly test for a JNK requirement in immune signaling, we induced JNK mutant clones in the fat body of larvae. Strikingly, *diptericin*, *attacin*, *Metchnikowin*, and *drosomycin* expression was lost in the mutant tissue.

We conclude that the JNK pathway is required to mediate dTAK1 signaling during the *Drosophila* immune response. Furthermore, we propose a model where the JNK and NF-κB signaling are both required to activate antimicrobial peptide gene expression during the immune response in the *Drosophila* fat body.

## Results

### *Identification of a novel allele of dTAK1*

We undertook an EMS mutagenesis screen to isolate adult viable mutations on the X-chromosome that impaired the expression of *diptericin* in response to bacterial challenge. In addition to immune response defects, one mutation, *fb(x)179*, exhibited a weakly penetrant maternal effect phenotype that was reminiscent of, and enhanced by, single maternal alleles of JNK pathway components. Recombination mapping, complementation testing, and sequencing indicated that this mutation fell within the *Drosophila TGFβ-activated kinase 1* (*dTAK1*) gene (Figure 1, and not shown) revealing a glycine to aspartate missense mutation in the ATP binding motif in the kinase subdomain I region, which renders the protein an inactive kinase (Figure 1A). Based on these results, we henceforth will refer to *fb(x)179* as $dTAK1^{179}$.

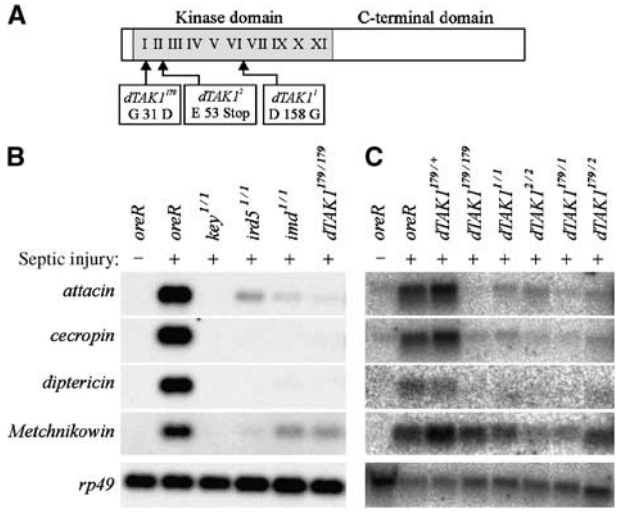


**Figure 1** *dTAK1* mutations block the expression of antibacterial peptide genes. (**A**) Diagram of genetic lesions in *dTAK1* alleles used in this study. Roman numerals represent the kinase subdomains. (**B**) Northern blot comparison of $dTAK1^{179}$ with mutations in Imd pathway genes. (**C**) Northern blot analysis of *dTAK1* mutants and complementation test. Adult flies of the indicated genotypes were infected with *E. coli* and then incubated for 12 h (B) or overnight (C) at 25°C. RNA was then prepared and analyzed as described in Materials and methods.

$dTAK1^{179}$ mutant animals failed to express Relish-dependent peptides in response to *Escherichia coli* infection (Figure 1B and C). We compared $dTAK1^{179}$ with other imd pathway mutants. $dTAK1^{179}$ mutants behaved like *imd* mutants and showed strongly reduced expression of Gram$^-$ antimicrobial genes like attacin, cecropin, and diptericin and a more modest reduction in *Metchnikowin* expression. We also observed reduced *defensin*, *drosocin*, and slightly reduced *drosomycin* expression (data not shown). These expression profiles are comparable to other *dTAK1* alleles (Vidal *et al*, 2001) and complementation tests indicated that $dTAK1^{179}$ behaves like a null (Figure 1C).

### *Relish is activated normally in dTAK1 flies and larvae*

Ectopic expression of *dTAK1*, as well as *imd* and *dredd*, constitutively activates *diptericin* expression (Vidal *et al*, 2001). Like the mammalian NF-κB proteins p100 and p105, Relish is a compound protein with an N-terminal DNA-binding Rel homology domain and a C-terminal inhibitory ankyrin-repeat domain. Signaling via the IKK complex results in Dredd-dependent cleavage of full-length Relish, REL-110, into a nuclear-active N-terminal fragment, REL-68, and a cytoplasmically stable C-terminal fragment, REL-49 (Stöven *et al*, 2000, 2003). In contrast to current models, Western blot analysis using an antibody specific for the C-terminal domain of Relish revealed that processing of endogenous Relish protein was intact in all three *dTAK1* mutant strains including the protein null (Figure 2A). Processing did not occur in $key^1$ mutant animals (Supplementary Figure 1; also see Stöven *et al*, 2003). We examined the intracellular localization of the REL-68 fragment in control and *dTAK1* mutant fat body tissue using an antibody specific for the Rel homology domain (Stöven *et al*, 2000). Consistent with the above results, we detected an enrichment of REL-68 in the fat body nuclei of infected *dTAK1* larvae just as in control animals (Figure 2B).

In further support of the finding that Relish cleavage and nuclear localization were normal in *dTAK1* mutant animals, we tested the binding of Relish to the promoters of antimicrobial peptide genes. *Drosophila* κB binding motifs have been defined that are sufficient for Relish protein binding (Stöven *et al*, 2000). Using a cecropinA1 κB sequence as probe, we performed electromobility shift assays (EMSAs) to determine if binding activity persisted in *dTAK1* mutant animals. We found κB binding activity (κBA) in protein extracts from *dTAK1* mutant animals, just as in control extracts (Figure 2C). We confirmed that this κBA was Relish by supershift with Relish-specific antibody and the loss of κBA in extracts from *Relish* mutant animals (Figure 2D).

We sought to test the association of Relish with endogenous promoters in *dTAK1* mutants. The analysis of Relish and association with endogenous promoter elements has been studied using the technique of chromatin immunoprecipitation (ChIP) in *Drosophila* S2 cells (Kim *et al*, 2005). We applied the ChIP technique to *Drosophila* larval fat body cells and compared samples from naïve and infected wild type and immune challenged *dTAK1* and *IKKγ* mutants (Figure 2E). Antibodies specific for the N-terminal domain

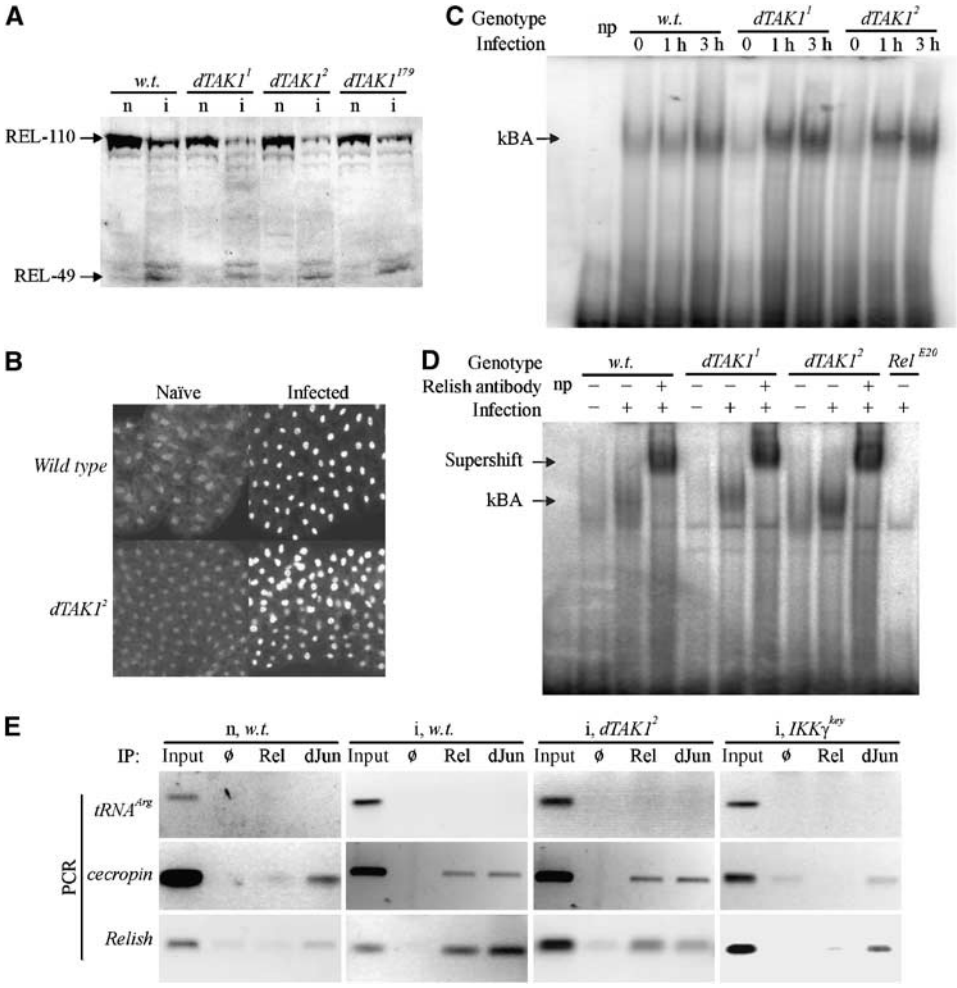


**Figure 2** Relish activation is normal in *dTAK1* mutant flies and larvae. (**A**) Relish is cleaved in *dTAK1* larvae. Protein extracts were prepared from naïve (n) or infected (i) wandering third-instar larvae of the wild-type (*w.t.*, Canton S), $dTAK1^{1}$, $dTAK1^{2}$, and $dTAK^{179}$ backgrounds and analyzed by Western blotting with a monoclonal antibody specific for the C-terminal part of Relish, REL-49 (Stöven *et al*, 2000). (**B**) Relish is translocated to the nucleus in $dTAK1^{2}$ mutant larvae. Fat body from wild-type (Canton S) and $dTAK1^{2}$ third–instar larvae was fixed and the N-terminal part of Relish was visualized as described in Materials and methods. (**C**) κBA is present in *dTAK1* mutant flies. Flies were challenged with a mixture of *M. luteus* and *Enterobacter cloacae* for the times indicated before preparing nuclear extracts and performing EMSA. A lane with no protein (np) is included. (**D**) Relish is a component of the κBA in *TAK1* mutant flies. The κBA in both wild-type and *dTAK1* mutant nuclear extracts is shifted by incubation with Relish-specific antibody and no κBA is observed in extracts from *Relish* mutant flies. The np lane contains no added protein extract. (**E**) Relish binds to target promoters in *dTAK1* mutant fat body. Chromatin immunopurification was performed on extracts from fat bodies of naïve (n) *w.t.* (Oregon R) and infected (i) *w.t.*, $dTAK1^{2}$, and $IKK\gamma^{key}$ animals using antibodies-specific for the Relish N-terminus (Rel) or dJun proteins or a blank (Ø) precipitation as indicated. Primers corresponding to sequences proximal to the promoters of $tRNA^{Arg}$ (as negative control), *cecropinA*, and *Relish* genes were used to detect the presence of these sequences by PCR. Pre-IP input samples were used at 1000-fold dilution to provide a comparable positive control signal.

of Relish (Rel) or the *Drosophila* Jun (dJun) protein were used to IP the endogenous proteins and associated chromosomal sequences, and primers corresponding to promoters of the $tRNA^{Arg}$, *CecropinA*, and *Relish* genes were used to detect co-precipitated DNA by PCR. The $tRNA^{Arg}$ promoter does not have any discernable NF-κB or dJun binding sites and thus served as a negative control (Figure 2E, row 1). In comparison, *cecropinA* and *Relish* sequences were detected in both Rel and dJun IP samples from infected wild-type larvae. Strikingly, *cecropinA* and *Relish* were also detected in infected *dTAK1* mutant samples by both Rel and dJun IP (Figure 2E, column 3). Importantly, *cecropinA* and *Relish* were not readily detected in naïve, wild type or infected, $IKK\gamma^{key}$ samples by Rel IP, but were detected by dJun IP, reflecting the requirement for infection and $IKK\gamma^{key}$ function for Relish activation (Figure 2E, rows 1 and 2). We detected *cecropinA* and *Relish* in all dJun IP samples, regardless of experimental conditions, consistent with dJun being always nuclear and associated with promoters, even in the absence of signal (Weiss *et al*, 2003). In summary, these data indicate that the cleavage, nuclear translocation, and promoter binding activity of Relish persist in *dTAK1* mutant animals, but are not sufficient for the expression of the antimicrobial peptide genes.

### *JNK but not Relish signaling components mediate dTAK1 function*

As dTAK1 has been implicated in JNK signaling during developmental patterning and apoptosis (Takatsu *et al*, 2000; Mihaly *et al*, 2001; Igaki *et al*, 2002), we tested downstream JNK signaling components in the context of the innate immune response. The *Drosophila* gene *hemipterous* (*hep*)

 

encodes a JNK kinase and can act downstream of dTAK1 function. For example, null mutants in *hep* suppressed the planar cell polarity defects of ectopic *dTAK1* expression in the *Drosophila* eye (Mihaly *et al*, 2001). If dTAK1 acts in JNK signaling rather than imd signaling during the immune response, then activated forms of downstream JNK pathway components might suppress the *dTAK1* mutant phenotype. We expressed an activated form of *Hep* (*Hep.CA*) in *dTAK1* mutant and control flies in the presence and absence of infection (Figure 3A). Unlike *imd*, *dredd*, and *dTAK1*, expression of *Hep.CA* itself caused no constitutive expression of *diptericin*. However, expression of activated *Hep.CA* in $dTAK1^{2}$ but not $IKK\gamma^{key}$ mutant flies resulted in *diptericin* expression, but only in response to infection (Figure 3A).

As noted previously, overexpression of the *Drosophila* caspase Dredd can induce the expression of *diptericin* in the absence of infection (Vidal *et al*, 2001), presumably owing to ectopic cleavage of Relish. We confirmed that overexpression of *Dredd* could induce expression of *diptericin* and *attacin* in a wild-type background, but that this expression was suppressed in both $dTAK^{2}$ and $dTAK^{179}$ mutant animals (Figure 3B) consistent with a model that places dTAK1 downstream or parallel to Dredd. Overexpressed *Dredd* only weakly induced *drosomycin* expression. However, this low level of expression was still sensitive to loss of *dTAK1* (Figure 3B). Given that Dredd has been shown to play a crucial role in Relish processing (Stöven *et al*, 2000, 2003) and that Relish cleavage is normal in *dTAK1* mutant animals (Figure 2), dTAK1 does not act downstream of Dredd to activate Relish. An alternative role for Dredd has been proposed in the ubiquitin-mediated activation of dTAK1 and the dIKK complex (Zhou *et al*, 2005). Our data are consistent with this alternate Dredd function and do not exclude the possibility of either function. Nevertheless, we favor a model in which dTAK1 acts in the JNK signaling pathway in parallel to IKK signaling and Relish cleavage to control *diptericin* induction.

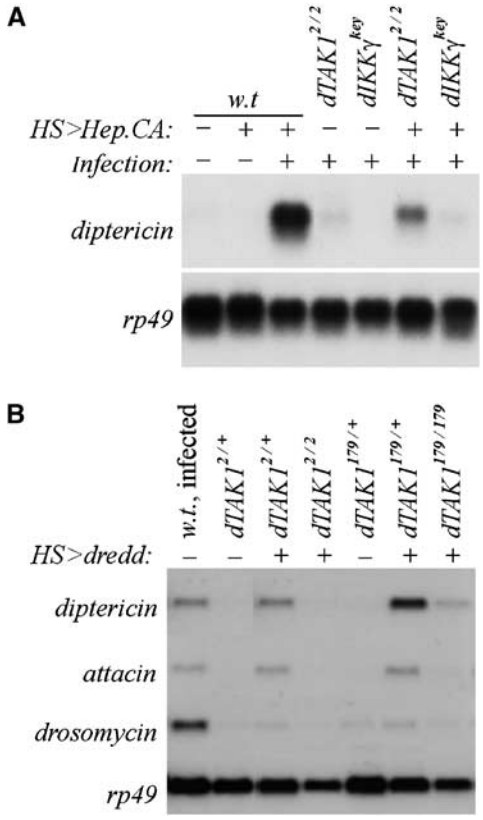


**Figure 3** JNK but not Relish signaling components mediate dTAK1 function. (**A**) Flies of the indicated genotypes were either untreated (−) or subjected to heat shock (+) to active *HS-GAL4*-driven *UAS-Hep.CA* expression, and infected with a mixture of *M. luteus and E. coli*(+). (**B**) Wild-type Oregon R flies were infected overnight and RNA was prepared. *HS-GAL4* and *UAS-Dredd* transgenes were crossed into $dTAK1^{2}$ and $dTAK^{179}$ mutant backgrounds. Flies of the indicated genotypes were either untreated (−) or heat shocked (+) and analyzed by Northern blot (see Materials and methods).

### *puc, an inhibitor of JNK signaling, suppresses antimicrobial peptide expression*

As an independent test of the role of JNK signaling in the *Drosophila* immune response, we overexpressed the phosphatase Puc in the fat body. Puc is a negative feedback regulator of the JNK pathway that inactivates JNK function (Martin-Blanco *et al*, 1998). Strikingly, Puc overexpression in the fat body reduced antimicrobial gene expression upon infection by as much as 90% (Figure 4A). Interestingly, Puc suppressed more than strictly Relish-dependent peptides as both *metchnikowin* and *drosomycin* expression was also reduced in these animals (see also Discussion).

Puc phosphatase activity is specific for JNKs and expression of Puc has no known effect on other kinases or pathways, and thus is not anticipated to have any inhibitory effect on IKK signaling (Martin-Blanco *et al*, 1998). To test this directly, we overexpressed Puc in the fat body of flies and examined Relish cleavage by Western analysis. Relish cleavage was normal in Puc-expressing flies as compared with siblings that lacked the YP1-Gal4 driver (Figure 4B). For comparison, no Relish cleavage was detected in flies mutant for the Dredd caspase (Figure 4B). This corroborates that the function of dTAK1 is independent of, and parallel to, Relish, and that together they have a combinatorial influence on downstream events. Together, these data suggest that JNK signaling is required in the fat body for the normal expression of antimicrobial genes and dTAK1 function.

### *Antimicrobial peptide gene expression is blocked in JNK mutant clones in vivo*

JNKs as a kinase family are well conserved in both structure and in choice of phosphorylation targets. JNK signaling components are also expressed in most tissues. We detected by, RT–PCR, expression of *bsk* (*dJNK*) and *hep* in the larval fat body (data not shown). We also detected dJun and Bsk proteins in the larval fat body, confirming that JNK proponents are present in this tissue (data not shown). As *JNK* mutations are embryonic lethal, we examined the immune response in FLP/FRT-induced *JNK* mutant clones (using the null $bsk^{2}$ and $bsk^{170b}$ alleles) in the fat body of infected larvae (Theodosiou and Xu, 1998; Manfruelli *et al*, 1999). We probed for the endogenous gene expression of antimicrobial peptides directly by *in situ* hybridization. Consistent with our hypothesis, we found that $JNK^{-/-}$ clones failed to express *diptericin* and *attacin* in response to infection (Figure 5A and data not shown). Consistent with the Puc expression data (Figure 4), *JNK* mutant tissue also showed reduced expression of *metchnikowin* and *drosomycin* (Figure 5B and C).

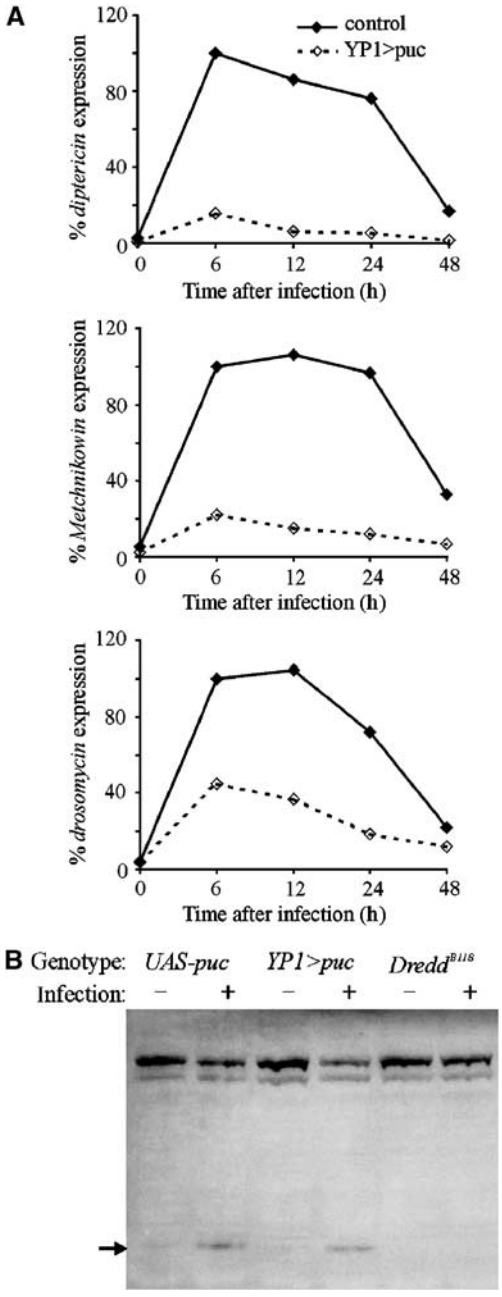


**Figure 4** Overexpression of *puc*, an inhibitor of JNK signaling, suppresses antimicrobial peptide expression. (**A**) *YP1-GAL4*, an adult fat body driver, and *UAS-puc* transgenic lines were crossed. Control flies (solid lines) that carried only the *YP1-GAL4* transgene or *YP1>puc* female flies that carried both transgenes (dashed lines) were analyzed for expression levels of antimicrobial peptide genes after bacterial infection using Northern hybridization. All data were normalized to rp49 signal and presented as percent signal intensity relative to the signal at 6 h, arbitrarily set to 100%. Almost complete elimination of *diptericin* was observed, and *Metchnikowin* and *drosomycin* were also reduced. (**B**) Overexpression of *puc* does not block Relish cleavage. Flies were crossed as in (A) and females that carried either the *UAS-puc* transgene alone or both the *YP1-GAL4* and *UAS-puc* (*YP1>puc*) were infected as indicated. For comparison, extracts from naïve and infected $Dredd^{B118}$ flies were included that show no Relish cleavage.

We also performed similar clonal analyses with alleles of *dJun* and *misshapen* (*msn*, encoding a *Drosophila* MAPKKKK). Using either the $msn^{102}$ or $msn^{172}$ allele, we could only recover very few largely single cell clones. Although *diptericin* expression was absent in these cells, the mutant cells did not quite appear normal and in some cases were partially excluded from the surrounding tissue, rendering interpretation difficult (Figure 5D). *Drosomycin* expression was also reduced in mutant cells (not shown). Similarly for *dJun*, we could not recover mutant tissue for some alleles ($dJun^2$ or $dJun^3$, although we could identify GFP-bright twin spots). We recovered rare clones of the $dJun^1$ allele and found that *attacin* and *drosomycin* expression was reduced (Figure 5E and F). In contrast to *dTAK1* and *bsk*, we infer from these results that *dJun* and *msn* may be essential for viability of fat body tissue. Nevertheless, these results are consistent with a role for *dJun* and *msn* in the immune response in the fat body. In agreement with these data, RNA interference (RNAi) knockdown of *kayak/dFos*, *msn*, or *hep* in S2 cells can also block *attacin* and *drosomycin* expression (Kallio *et al*, 2005).

To control for the health and responsiveness of the mutant fat body cells, we looked at *rp49* RNA expression levels and *Drosophila* STAT (dSTAT) protein levels and nuclear localization in response to infection (Agaisse *et al*, 2003). Unlike the antimicrobial genes, *rp49* expression was not altered in *bsk* mutant tissue (Figure 5G). Basal dSTAT protein levels were unaltered across *bsk* mutant clone borders (Figure 5H). Upon infection, dSTAT protein localized normally to the nuclei in *bsk* mutant tissue (Figure 5I).

Based on the suppression of the immune response by *puc* overexpression and in *bsk* mutant clones, we conclude that JNK signaling is an essential component of the *Drosophila* immune response in the fat body (see also below). Taken together with the rescue of *dTAK1* mutants by transgenic JNKK expression, our data indicate that dTAK1 signals through JNK in the immune response.

## Discussion

The function of TAK1 in vertebrates has remained enigmatic. It was originally identified as a TGFβ-activated kinase, hence the name, in mammalian cell culture assays (Shibuya *et al*, 1996; Behrens, 2000). However, follow-up work in multicellular contexts and *in vivo* analyses in vertebrates, *Caenorhabditis elegans*, and *Drosophila* have shown no clear link to TGFβ signaling, but rather suggest a role for TAK family kinases in JNK activation or as upstream activators of Nemo-like kinases (Behrens, 2000). In mammalian systems, TAK1 is one of a number of kinases that can activate IKK complexes and, consequently, NF-κB signaling *in vitro*. *In vitro* studies of human cells have shown that targeting of *TAK1* by RNAi reduces NF-κB activation by TNFα and IL-1 stimulation (Takaesu *et al*, 2003). Recent studies using fibroblasts derived from *TAK1* mutant mouse embryos and mice with a B-cell-specific deletion of *TAK1* showed that JNK activation was impaired in response to all stimuli tested in *TAK1* mutant cells (Sato *et al*, 2005; Shim *et al*, 2005). Although NF-κB activation was impaired in response to stimulation by IL-1β, TNF, and TLR3 and TLR4 ligands, NF-κB activation by B-cell receptor or LT-β stimulation remained intact, suggesting a specific role for TAK1 upstream of IKKβ and JNK, but not IKKα (Sato *et al*, 2005; Shim *et al*, 2005). Interestingly, IKKα activation leads to the phosphorylation and processing of NF-κB2 from the p100 to the active p52 form (Hayden and Ghosh, 2004), reminiscent of Relish activation in *Drosophila*.

 

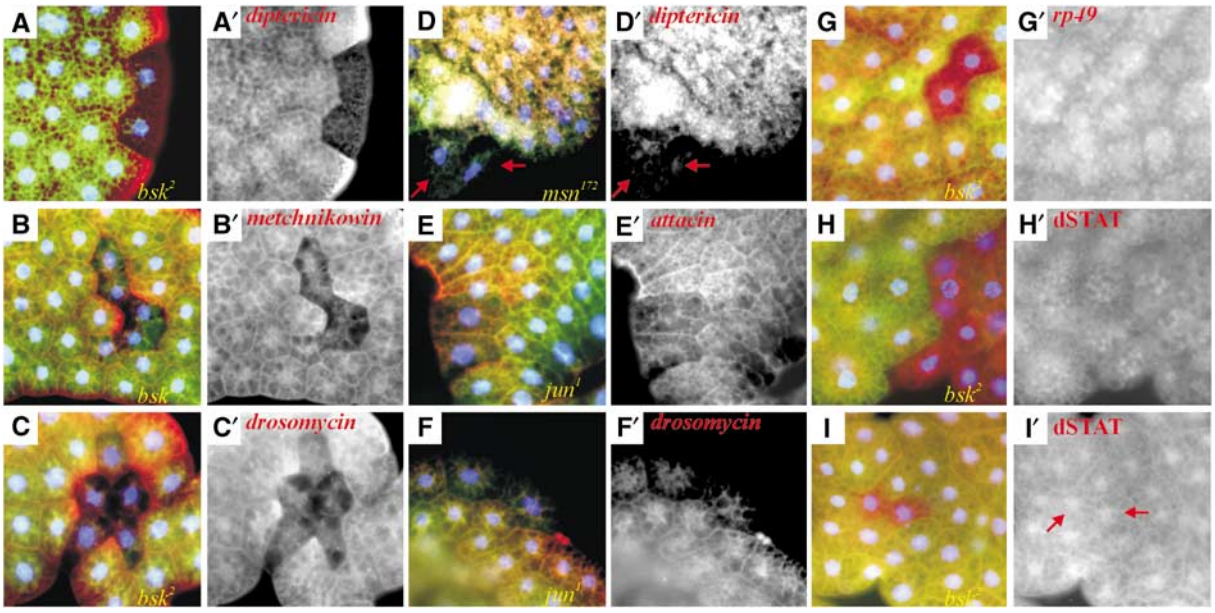


**Figure 5** JNK pathway mutations block antimicrobial peptide gene expression in larval fat body tissue. Mosaic $bsk^2$, $msn^{172}$, and $jun^1$ mutant larval fat body clones were each generated individually and analyzed as described (see Materials and methods). Nuclear Hoechst staining is in blue. Mutant clonal tissue is marked by the absence of GFP in green. Genotypes are noted in the lower right of three-color panels. (**A**) *diptericin* expression (red) is absent in $bsk^2$ clones (all clones analyzed, >15, showed the same effect). (**A′**) *diptericin* expression shown in single channel. (**B**) *Metchnikowin* is lost in $bsk^2$ mutant clones (16 clones analyzed, all showing the same effect) and (**C**) *drosomycin* expression (red) is lost in $bsk^2$ mutant clones (again all out of 10 clones analyzed). (**D**) *diptericin* expression (red) is absent in $msn^{172}$ clones (all of five clones analyzed, out of over 300 dissected larvae). Note in this example how the two mutant cells seem to be excluded from the fat body (red arrows). (**E**) *attacin* (red) and (**F**) drosomycin expression (red) is reduced in $dJun^1$ mutant cells (two clones analyzed). (**G**) *rp49* expression (red) is unimpaired in $bsk^2$ mutant clones (seven clones analyzed). (**H**) dSTAT protein expression is normal in naïve $bsk^2$ mutant clones. (**I**) dSTAT protein is nuclear in infected $bsk^2$ mutant fat body cells (red arrows in (I′), five clones analyzed). Red channels are shown in (**B′, C′, D′, E′, F′, G′, H′, I′**).

Biochemical analyses in mammalian systems have demonstrated that TAK1 functions in multimeric protein complexes that can include TAB1, TAB2, and different TRAF proteins. The exact composition of these complexes seems to determine TAK1 responsiveness and downstream effects (Takaesu *et al*, 2003; Hayden and Ghosh, 2004, and references therein). In the fly, genetic studies found an interaction between *dTRAF1* and *dTAK1* in the activation of JNK signaling and apoptosis (Cha *et al*, 2003). Gain- and loss-of-function analyses indicate that *dTRAF2*, but not *dTRAF1*, is necessary for the activation of Relish-dependent gene expression; however, no interaction between *dTRAF2* and *dTAK1* in the activation of antimicrobial peptides was reported (Cha *et al*, 2003).

### *In vivo versus in vitro studies*

Genome-wide analyses that examined *in vivo* responses in *Drosophila* identified *dJun* and *puc* as genes potentially regulated by Toll and Imd signaling, suggesting a cross-regulation between these pathways and the JNK signaling pathway (De Gregorio *et al*, 2002). A study recently reported that RNAi knockdown of *kayak*, *msn*, *hep*, or *aop* blocked *E. coli*-induced *attacin* and *drosomycin* expression in S2 cells (Kallio *et al*, 2005). Furthermore, in related studies, they also observed that, although dTAK1 RNAi-treated S2 cells failed to express an *attacin* reporter gene, Relish cleavage and nuclear localization remained intact in these cells (Kleino *et al*, 2005). Other RNAi analyses in S2 cells concluded that JNK signaling did not have a significant role in antimicrobial peptide gene expression (Boutros *et al*, 2002; Silverman *et al*, 2003; Park *et al*, 2004). However, RNAi against *hep* or *bsk* seemed to partially block antimicrobial peptide induction, especially of *attacin* and *cecropinA* (Silverman *et al*, 2003) and, accordingly, *attacinD* levels were lower in microarrays when the JNK pathway was blocked (Boutros *et al*, 2002). Our results confirm a positive role for JNK signaling in the antimicrobial peptide response *in vivo*.

### *Integration of JNK and NF-κB signaling*

The placement of dTAK1 function upstream of JNK, rather than IKK, requires a remodeling of the signaling pathways that activate the antimicrobial peptide genes (Figure 6). Earlier models were based on studies that showed that *dTAK1* mutations blocked the constitutive activation of *diptericin* by Imd overexpression (Vidal *et al*, 2001). In turn, *IKK* mutations blocked dTAK1-induced *diptericin* expression. One interpretation of these data places IKK directly downstream of dTAK1. However, if the activation of both JNK and IKK signaling pathways is required, then a disruption in either branch would be sufficient to suppress any upstream activation.

Overexpression of dTAK1 is sufficient to induce antimicrobial peptide expression (Vidal *et al*, 2001). However, dTAK1 is an extremely potent activator of JNK signaling and apoptosis (Takatsu *et al*, 2000; Mihaly *et al*, 2001), and overexpression of dTAK1 could activate proteins that are not normal phosphorylation targets. Based on RNAi studies in S2 cells, *dTAK1* is required for dIKK complex-dependent phosphorylation of Relish *in vitro* (Silverman *et al*, 2003). This could reflect a stringent requirement for dTAK1 in blood cell-derived S2 cells that is different in fat body tissue.

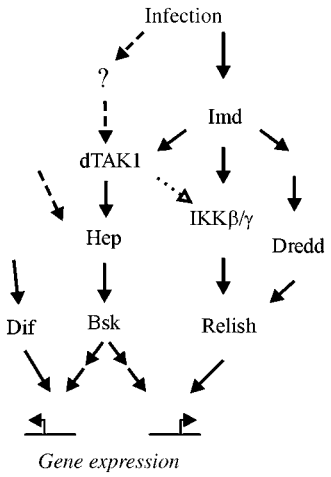


**Figure 6** Integration of our data into current models, initially based on Brennan and Anderson (2004). Dotted arrows represent potentially pleiotropic signaling that may occur owing to overexpression. Dashed arrows represent hypothetical signaling events that could initially activate JNK signaling during infection or mediate signaling independently of dTAK1, such as during Dif-controlled *drosomycin* expression. The question mark (?) represents our speculation that other receptor signaling events, such as TNFR activation, also modulate the innate immune response in the *Drosophila* fat body.

The new model (Figure 6) would predict that overexpression of the Dredd caspase would be insufficient to activate fully the antimicrobial peptides in *dTAK1* mutant animals and this is indeed the case (Figure 3B). Overexpression of Dredd may be sufficient to induce antimicrobial peptide gene expression in a wild-type background because of inadvertent JNK pathway activation by ectopic caspase activity or by the heat-shock protocol itself (Gibson and Perrimon, 2005). Alternatively, an additional role for Dredd has been proposed in the ubiquitin-mediated activation of dTAK1 and the dIKK complex (Zhou *et al*, 2005). The suppression by *dTAK1* mutants of ectopic *Dredd* expression is consistent with this model as well, and does not distinguish between the two potential functions of Dredd. Our data are consistent with a model that places dTAK1 activity in a pathway parallel to the functions of IKK and Relish and in which both these pathways are required for the activation of antibacterial peptide genes such as *diptericin* and *attacin*.

Promoter analyses of most antimicrobial peptide genes have not revealed any obvious binding sites for activator protein-1 (AP-1) complexes, the Jun/Fos heterodimer, and transcriptional mediator of JNK signaling (Kadalayil *et al*, 1997; Petersen *et al*, 1999; Senger *et al*, 2004). However, AP-1 binding sites can be quite diverse and are not always predictable directly from DNA sequence. Nevertheless, a recent study identified a functional AP-1 binding site in the *attacinA* promoter (Kim *et al*, 2005). Their data suggest that AP-1 binding represses *attacinA* transcription by recruiting histone deacetylase 1 (dHDAC1) to the promoter. In contrast, in mammalian studies, c-Jun function is itself repressed by association with HDAC3. This repression is relieved upon JNK signaling (Weiss *et al*, 2003). A similar mechanism may be employed in the *Drosophila* fat body. Accordingly, the sustained expression of *attacin* and other antimicrobial peptide genes *in vivo* would require an activation (or de-repression) of AP-1 function at the onset of the immune response. Such positive cooperation between AP-1 and NF-κB transcription factors was also seen in molecular studies of the human β-defensin-2 promoter (Wehkamp *et al*, 2004).

AP-1-dependent gene expression is normally rapid. Thus, if AP-1 activity is not directly required for *diptericin* expression, it could act indirectly through the activation of other genes. Alternatively, JNK could phosphorylate some targets other than the AP-1 complex proteins Jun and Fos. In mammalian studies, it has been shown that JNK can phosphorylate, and thereby inhibit, Insulin Receptor Substrate-1 (Lee *et al*, 2003). However, the recent finding that RNAi against *kayak/dFos* can block antimicrobial peptide expression and our *dJun* loss-of-function studies *in vivo* suggest that JNK does indeed signal through AP-1 to control expression of these genes (Kallio *et al*, 2005).

It is intriguing that overexpressed Puc not only blocked Relish-dependent antimicrobial peptide gene expression, but it also strongly blocked *drosomycin* expression, which is not true in *dTAK1* mutants (Vidal *et al*, 2001). This suggests that JNK or JNK-related proteins, for example, p38a, p38b, and MPK2, may also be important for other aspects of the immune response, for example, the Toll/Dif-dependent antimicrobial genes (Sluss *et al*, 1996; Han *et al*, 1998). Our clonal analysis of *JNK* mutant tissue confirms that JNK is required not only for the expression of $Gram^-$-specific peptides *diptericin* and *attacin*, but also for *Metchnikowin* ($Gram^+$/fungal specific) (Levashina *et al*, 1995) and *drosomycin* (fungal specific) (Fehlbaum *et al*, 1994). Mutations in *dTAK1* had less of an impact on *Metchnikowin* or *drosomycin* expression than on *attacin*, for example. Furthermore, reduced dJun activation occurred in dTAK1 mutant animals, indicating that other upstream kinases may be involved in the control of these genes. JNK is a member of a large family of mitogen-activated protein kinases (MAPKs). In the fly, there are at least five MAPKKKs, four MAPKKs, and five MAPKs, and so the potential redundancies are many. If these other proteins contribute to the immune response, how they do so has yet to be tested in genetic loss-of-function *in vivo* studies in the fat body.

How JNK and NF-κB signals integrate to positively control gene expression is a critical question. Here, we have demonstrated that both are required for the expression of a particular set of immune responsive genes *in vivo*. Through the use of *Drosophila* genetics, we should be able to identify novel immune response genes that are controlled cooperatively by JNK and NF-κB signaling. From promoter analysis of these genes, we may be able to predict additional genes that are important for other biological processes. Both the JNK and NF-κB signaling pathways have been implicated many times in many different contexts. Continued analysis in *Drosophila* may lead to a general understanding of their roles in normal biological processes and developmental malignancies.

## Materials and methods

### *Drosophila strains*

Oregon R, Canton S, or *diptericin-lacZ(X)* (Reichhart *et al*, 1992), the parental strain of the EMS-induced $dTAK1^{179}$ mutation, was used as a wild-type control. $dTAK1^{1}$ and $dTAK1^{2}$ were described previously (Vidal *et al*, 2001) as were $ird5^{1}$ (Lu *et al*, 2001), $key^{1}$ (Silverman *et al*, 2000), $imd^{1}$ (Lemaitre *et al*, 1995), $Dredd^{B118}$ (Leulier *et al*, 2000), $bsk^{2}$, $bsk^{170b}$ (Sluss *et al*, 1996), $msn^{102}$, $msn^{172}$ (Treisman *et al*, 1997), $dJun^{1}$, $dJun^{3}$ (Kockel *et al*, 1997),

 

and *dJun*[2] (Nusslein-Volhard *et al*, 1984). For ectopic expression of *hemipterous*, *puckered*, and *dredd*, we employed the UAS/GAL4 system by crossing *UAS-Hep.CA* (Adachi-Yamada *et al*, 1999), *UAS-puc* (Martin-Blanco *et al*, 1998), and *UAS-dredd* (Vidal *et al*, 2001) to either *YP1-GAL4*, specific for female fat body (Vidal *et al*, 2001), or to *Heat Shock-GAL4* and analyzed the offspring.

### *Northern analysis*

To test antimicrobial gene expression, wandering third-instar larvae and adults were infected overnight with *E. coli* as described (Wu and Anderson, 1998) and total RNA extracted using Trizol Reagent (Invitrogen). A 5–10 μg portion of RNA was loaded per lane and then transferred to Hybond nylon membrane. Random prime labeled probes (Boehringer) were made for each of the antimicrobial peptides and rp49. For *HS>dredd* experiments, flies of the indicated genotypes were either untreated (−) or heat shocked (+) at 37°C for 1 h, rested overnight, heat shocked a second time, and rested 6 h. For *HS>Hep.CA* experiments, flies of the indicated genotypes were either untreated (−) or subjected to heat shock and/or infection with *E. coli*(+). Infection was just prior to a single 1 h heat shock at 37°C. RNA was prepared after an overnight incubation.

### *Western analysis and immunohistochemistry*

Analysis of endogenous Relish protein was performed as described (Stöven *et al*, 2000). Preparation of larval fat body and immunohistochemical detection of Relish were carried out as previously described (Stöven *et al*, 2000) using an affinity-purified rabbit polyclonal antibody specific for the Rel homology domain and anti-rabbit IgG/Cy2 conjugate (Jackson ImmunoResearch). A rabbit polyclonal antibody specific for dSTAT was likewise used to detect dSTAT protein in larval fat body (Chen *et al*, 2002). Preparations of nuclear extracts were carried out as described (Uvell and Engström, 2003).

### *EMSA*

EMSAs were carried out as described (Uvell and Engström, 2003) using the Cecropin A1 κB sequence as probe: tcgagacacGGGGA TTTTTgcac. For supershift experiments, extracts were incubated with 1 μl of relish antiserum (Stöven *et al*, 2000) before the addition of probe.

### *ChIP*

ChIP was carried largely as described for S2 cells (Kim *et al*, 2005). For a given experiment, 80–100 larvae were collected for direct processing or infected with a *Micrococcus luteus*/*E. coli* mixture for 10–15 min. Larvae were then dissected and fixed in 1% formaldehyde/PBS for 10 min, washed, and then fat body was dissected away from gut and integument and placed directly into lysis solution and then subjected to sonication. Immunopurification was performed with rabbit polyclonal antibodies specific for the Rel homology domain (Stöven *et al*, 2000) or dJun (Bohmann *et al*, 1994). The following primers were used for PCR detection of promoter sequences: *tRNA*$^{Arg}$-forward, 5′-CACAAGCAAACAGCAG AAGTAAAC-3′, *tRNA*$^{Arg}$-reverse, 5′-CATCGGTTTTATACCTCAAGAT GC-3′, *cecropinA*-forward, 5′-GATTGTTCCCTAGATGTGCAG-3′, *cecropinA*-reverse, 5′-GCGACTGATGACTGCGATAC-3′, *Relish*-forward, GAACCGTAGTTTCCGTGAAAAGCT-3′, *Relish*-reverse, 5′-GCAGCGA ATCGGGGAACTTTAGTG-3′.

### *In situ hybridization of mosaic clones*

Mosaic clones were generated from the following crosses, *bsk*$^{2}$*: w, HS-FLP*$^{122}$*; bsk*$^{2}$*FRT40A/CyO X w, HS-FLP*$^{122}$*; ubi-GFP FRT40A/CyO, msn*$^{172}$*: w, HS-FLP*$^{122}$*; msn*$^{172}$*FRT80/TM6B X w, HS-FLP*$^{122}$*; ubi-GFP FRT80, jun*$^{1}$*: w, HS-FLP*$^{122}$*; jun*$^{1}$*FRT42/SM5a:TM6B X w, HS-FLP*$^{122}$*; ubi-GFP FRT42*. Embryos were collected 2–4 h after egg laying, incubated for 2 h, and then heat shocked for 1 h at 37°C and allowed to develop to third instar at 25°C. GFP$^{+}$ larvae were sorted by fluorescence microscopy, infected with *E. coli*, and then allowed to rest for 3 h before dissection and overnight fixation in 8% formaldehyde/0.1% Tween 20/PBS at 4°C. *In situ* hybridization and subsequent visualizations with DIG-labeled RNA *diptericin* probe was carried out as described (Hauptmann, 2001; Agaisse *et al*, 2003). Sheep anti-DIG (Boehringer), biotinylated donkey anti-sheep (Jackson), and TSA TRITC (Perkin-Elmer) reagents were used to visualize *in situ* probes. FITC-labeled goat anti-rabbit (Jackson) and rabbit anti-GFP (Abcam) antibodies were used to visualize GFP. Nuclei were stained with Hoechst reagent. Analysis of diptericin expression induced by a mixture of *M. luteus* and *Erwinia carotovora* and overnight incubations post-infection yielded results similar to Figure 5A.

### *Supplementary data*

Supplementary data are available at *The EMBO Journal* Online.

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## References

Adachi-Yamada T, Fujimura-Kamada K, Nishida Y, Matsumoto K (1999) Distortion of proximodistal information causes JNK-dependent apoptosis in *Drosophila* wing. *Nature* **400:** 166–169

Agaisse H, Petersen UM, Boutros M, Mathey-Prevot B, Perrimon N (2003) Signaling role of hemocytes in *Drosophila* JAK/STAT-dependent response to septic injury. *Dev Cell* **5:** 441–450

Behrens J (2000) Cross-regulation of the Wnt signalling pathway: a role of MAP kinases. *J Cell Sci* **113:** 911–919

Bohmann D, Ellis MC, Staszewski LM, Mlodzik M (1994) *Drosophila* Jun mediates Ras-dependent photoreceptor determination. *Cell* **78:** 973–986

Boutros M, Agaisse H, Perrimon N (2002) Sequential activation of signaling pathways during innate immune responses in *Drosophila*. *Dev Cell* **3:** 711–722

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118:** 401–415

Brennan CA, Anderson KV (2004) *Drosophila*: the genetics of innate immune recognition and response. *Annu Rev Immunol* **22:** 457–483

Cha G-H, Cho KS, Lee JH, Kim M, Kim E, Park J, Lee SB, Chung J (2003) Discrete functions of TRAF1 and TRAF2 in *Drosophila melanogaster* mediated by c-Jun N-terminal kinase and NF-κB-dependent signaling pathways. *Mol Cell Biol* **23:** 7982–7991

Chen H-W, Chen X, Oh S-W, Marinissen MJ, Gutkind JS, Hou SX (2002) mom identifies a receptor for the *Drosophila* JAK/STAT signal transduction pathway and encodes a protein distantly related to the mammalian cytokine receptor family. *Genes Dev* **16:** 388–398

Dabbagh K, Lewis DB (2003) Toll-like receptors and T-helper-1/T-helper-2 responses. *Curr Opin Infect Dis* **16:** 199–204

De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B (2002) The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *EMBO J* **21:** 2568–2579

Dong C, Davis RJ, Flavell RA (2002) MAP kinases in the immune response. *Annu Rev Immunol* **20:** 55–72

Fehlbaum P, Bulet P, Michaut L, Lagueux M, Broekaert W, Hetru C, Hoffmann J (1994) Insect immunity. Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. *J Biol Chem* **269:** 33159–33163

Gibson MC, Perrimon N (2005) Extrusion and death of DPP/BMP-compromised epithelial cells in the developing *Drosophila* wing. *Science* **307:** 1785–1789

Han ZS, Enslen H, Hu X, Meng X, Wu IH, Barrett T, Davis RJ, Ip YT (1998) A conserved p38 mitogen-activated protein kinase pathway regulates *Drosophila* immunity gene expression. *Mol Cell Biol* **18:** 3527–3539

Hauptmann G (2001) One-, two-, and three-color whole-mount *in situ* hybridization to *Drosophila* embryos. *Methods* **23:** 359–372

Hayden MS, Ghosh S (2004) Signaling to NF-κB. *Genes Dev* **18:** 2195–2224

Hedengren-Olcott M, Olcott MC, Mooney DT, Ekengren S, Geller BL, Taylor BJ (2004) Differential activation of the NF-κB-like factors Relish and Dif in *Drosophila melanogaster* by fungi and Gram-positive bacteria. *J Biol Chem* **279:** 21121–21127

Igaki T, Kanda H, Yamamoto-Goto Y, Kanuka H, Kuranaga E, Aigaki T, Miura M (2002) Eiger, a TNF superfamily ligand that triggers the *Drosophila* JNK pathway. *EMBO J* **21:** 3009–3018

Kadalayil L, Petersen UM, Engström Y (1997) Adjacent GATA and kappa B-like motifs regulate the expression of a *Drosophila* immune gene. *Nucleic Acids Res* **25:** 1233–1239

Kallio J, Leinonen A, Ulvila J, Valanne S, Ezekowitz RA, Rämet M (2005) Functional analysis of immune response genes in *Drosophila* identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells. *Microb Infect* **7:** 811–819

Kim T, Yoon J, Cho H, Lee W-B, Kim J, Song Y-H, Kim SN, Yoon JH, Kim-Ha J, Kim Y-J (2005) Downregulation of lipopolysaccharide response in *Drosophila* by negative crosstalk between the AP1 and NF-κB signaling modules. *Nat Immunol* **6:** 211–218

Kleino A, Valanne S, Ulvila J, Kallio J, Myllymäki H, Enwald H, Stöven S, Poidevin M, Ueda R, Hultmark D, Lemaitre B, Rämet M (2005) Inhibitor of apoptosis 2 and TAK1-binding protein are components of the *Drosophila* Imd pathway. *EMBO J* **24:** 3423–3434

Kockel L, Zeitlinger J, Staszewski L, Mlodzik M, Bohmann D (1997) Jun in *Drosophila* development: redundant and nonredundant functions and regulation by two MAPK signal transduction pathways [published erratum appears in Genes Dev 1998 Feb 1;12(3):447]. *Genes Dev* **11:** 1748–1758

Lee YH, Giraud J, Davis RJ, White MF (2003) c-Jun N-terminal Kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. *J Biol Chem* **278:** 2896–2902

Lemaitre B, Kromer-Metzger E, Michaut L, Nicolas E, Meister M, Georgel P, Reichhart JM, Hoffmann JA (1995) A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the *Drosophila* host defense. *Proc Natl Acad Sci USA* **92:** 9465–9469

Leppèa S, Bohmann D (1999) Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. *Oncogene* **18:** 6158–6162

Leulier F, Rodriguez A, Khush RS, Abrams JM, Lemaitre B (2000) The *Drosophila* caspase Dredd is required to resist gram-negative bacterial infection. *EMBO Rep* **1:** 353–358

Levashina EA, Ohresser S, Bulet P, Reichhart J-M, Hetru C, Hoffmann JA (1995) Metchnikowin, a novel immune-inducible proline-rich peptide from *Drosophila* with antibacterial and antifungal properties. *Eur J Biochem* **233:** 694–700

Lu Y, Wu LP, Anderson KV (2001) The antibacterial arm of the *Drosophila* innate immune response requires an IkappaB kinase. *Genes Dev* **15:** 104–110

Manfruelli P, Reichhart JM, Steward R, Hoffmann JA, Lemaitre B (1999) A mosaic analysis in *Drosophila* fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. *EMBO J* **18:** 3380–3391

Martin-Blanco E, Gampel A, Ring J, Virdee K, Kirov N, Tolkovsky AM, Martinez-Arias A (1998) *puckered* encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*. *Genes Dev* **12:** 557–570

Medzhitov R, Janeway Jr CA (1998) An ancient system of host defense. *Curr Opin Immunol* **10:** 12–15

Mihaly J, Kockel L, Gaengel K, Weber U, Bohmann D, Mlodzik M (2001) The role of the *Drosophila* TAK homologue dTAK during development. *Mech Dev* **102:** 67–79

Moreno E, Yan M, Basler K (2002) Evolution of TNF signaling mechanisms. JNK-dependent apoptosis triggered by Eiger, the *Drosophila* homolog of the TNF superfamily. *Curr Biol* **12:** 1263

Nusslein-Volhard C, Wieschaus E, Kluding H (1984) Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. *Roux's Arch Dev Biol* **193:** 267–282

Park JM, Brady H, Ruocco MG, Sun H, Williams D, Lee SJ, Kato Jr T, Richards N, Chan K, Mercurio F, Karin M, Wasserman SA (2004) Targeting of TAK1 by the NF-κB protein Relish regulates the JNK-mediated immune response in *Drosophila*. *Genes Dev* **18:** 584–594

Petersen UM, Kadalayil L, Rehorn KP, Hoshizaki DK, Reuter R, Engström Y (1999) Serpent regulates *Drosophila* immunity genes in the larval fat body through an essential GATA motif. *EMBO J* **18:** 4013–4022

Reichhart JM, Meister M, Dimarcq JL, Zachary D, Hoffmann D, Ruiz C, Richards G, Hoffmann JA (1992) Insect immunity: developmental and inducible activity of the *Drosophila* Diptericin promoter. *EMBO J* **11:** 1469–1477

Rutschmann S, Jung AC, Hetru C, Reichhart JM, Hoffmann JA, Ferrandon D (2000a) The Rel protein DIF mediates the antifungal but not the antibacterial host defense in *Drosophila*. *Immunity* **12:** 569–580

Rutschmann S, Jung AC, Zhou R, Silverman N, Hoffmann JA, Ferrandon D (2000b) Role of *Drosophila* IKK gamma in a toll-independent antibacterial immune response. *Nat Immunol* **1:** 342–347

Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, Matsumoto K, Takeuchi O, Akira S (2005) Essential function for the kinase TAK1 in innate and adaptive immune responses. **6:** 1087–1095

Senger K, Armstrong GW, Rowell WJ, Kwan JM, Markstein M, Levine M (2004) Immunity regulatory DNAs share common organizational features in *Drosophila*. *Mol Cell* **13:** 19–32

Shibuya H, Yamaguchi K, Shirakabe K, Tonegawa A, Gotoh Y, Ueno N, Irie K, Nishida E, Matsumoto K (1996) TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. *Science* **272:** 1179–1182

Shim J-H, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee K-Y, Bussey C, Steckel M, Tanaka N, Yamada G, Akira S, Matsumoto K, Ghosh S (2005) TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways *in vivo*. *Genes Dev* **19:** 2668–2681

Silverman N, Zhou R, Erlich RL, Hunter M, Bernstein E, Schneider D, Maniatis T (2003) Immune activation of NF-kappaB and JNK requires *Drosophila* TAK1. *J Biol Chem* **278:** 48928–48934

Silverman N, Zhou R, Stoven S, Pandey N, Hultmark D, Maniatis T (2000) A *Drosophila* Ikappa B kinase complex required for Relish cleavage and antibacterial immunity. *Genes Dev* **14:** 2461–2471

Sluss HK, Han Z, Barrett T, Davis RJ, Ip YT (1996) A JNK signal transduction pathway that mediates morphogenesis and an immune response in *Drosophila*. *Genes Dev* **10:** 2745–2758

Stöven S, Ando I, Kadalayil L, Engström Y, Hultmark D (2000) Activation of the *Drosophila* NF-kappaB factor Relish by rapid endoproteolytic cleavage. *EMBO Rep* **1:** 347–352

Stöven S, Silverman N, Junell A, Hedengren-Olcott M, Erturk D, Engström Y, Maniatis T, Hultmark D (2003) Caspase-mediated processing of the *Drosophila* NF-kappa B factor Relish. *Proc Natl Acad Sci USA* **100:** 5991–5996

Stronach BE, Perrimon N (1999) Stress signaling in *Drosophila*. *Oncogene* **18:** 6172–6182

Takaesu G, Surabhi RM, Park K-J, Ninomiya-Tsuji J, Matsumoto K, Gaynor RB (2003) TAK1 is critical for IκB kinase-mediated activation of the NF-κB pathway. *J Mol Biol* **326:** 105–115

Takatsu Y, Nakamura M, Stapleton M, Danos MC, Matsumoto K, O'Connor MB, Shibuya H, Ueno N (2000) TAK1 participates in c-Jun N-terminal kinase signaling during *Drosophila* development. *Mol Cell Biol* **20:** 3015–3026

Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. *Annu Rev Immunol* **21:** 335–376

Theodosiou NA, Xu T (1998) Use of FLP/FRT system to study *Drosophila* development. *Methods* **14:** 355–365

Treisman JE, Ito N, Rubin GM (1997) misshapen encodes a protein kinase involved in cell shape control in *Drosophila*. *Gene* **186:** 119–125

Uvell H, Engström Y (2003) Functional characterization of a novel promoter element required for an innate immune response in *Drosophila*. *Mol Cell Biol* **23:** 8272–8281

Vercelli D (2003) Innate immunity: sensing the environment and regulating the regulators. *Curr Opin Allergy Clin Immunol* **3:** 343–346

Vidal S, Khush RS, Leulier F, Tzou P, Nakamura M, Lemaitre B (2001) Mutations in the *Drosophila* dTAK1 gene reveal a

 

conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses. *Genes Dev* **15:** 1900–1912

Wehkamp J, Harder J, Wehkamp K, Meissner BW-v, Schlee M, Enders C, Sonnenborn U, Nuding S, Bengmark S, Fellermann K, Schroder JM, Stange EF (2004) NF-κB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a novel effect of a probiotic bacterium. *Infect Immun* **72:** 5750–5758

Weiss C, Schneider S, Wagner EF, Zhang X, Seto E, Bohmann D (2003) JNK phosphorylation relieves HDAC3-dependent suppression of the transcriptional activity of c-Jun. *EMBO J* **22:** 3686–3695

Wu LP, Anderson KV (1998) Regulated nuclear import of Rel proteins in the *Drosophila* immune response. *Nature* **392:** 93–97

Zhou R, Silverman N, Hong M, Liao DS, Chung Y, Chen ZJ, Maniatis T (2005) The role of ubiquitination in *Drosophila* innate immunity. *J Biol Chem* **280:** 34048–34055