

Mutations in the *Drosophila dTAK1* gene reveal a conserved function for MAPKKKs in the control of rel/NF- κ B-dependent innate immune responses

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In mammals, TAK1, a MAPKKK kinase, is implicated in multiple signaling processes, including the regulation of NF- κ B activity via the IL1-R/TLR pathways. TAK1 function has largely been studied in cultured cells, and its *in vivo* function is not fully understood. We have isolated null mutations in the *Drosophila dTAK1* gene that encodes dTAK1, a homolog of TAK1. *dTAK1* mutant flies are viable and fertile, but they do not produce antibacterial peptides and are highly susceptible to Gram-negative bacterial infection. This phenotype is similar to the phenotypes generated by mutations in components of the *Drosophila* Imd pathway. Our genetic studies also indicate that dTAK1 functions downstream of the Imd protein and upstream of the IKK complex in the Imd pathway that controls the Rel/NF- κ B like transactivator Relish. In addition, our epistatic analysis places the caspase, Dredd, downstream of the IKK complex, which supports the idea that Relish is processed and activated by a caspase activity. Our genetic demonstration of *dTAK1*'s role in the regulation of *Drosophila* antimicrobial peptide gene expression suggests an evolutionary conserved role for TAK1 in the activation of Rel/NF- κ B-mediated host defense reactions.

[Key Words: Innate immunity; antimicrobial peptide; rel/NF- κ B; Toll; TAK1; caspase]

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Insects rely on multiple strategies to defend themselves against microbial infection: (a) they activate proteolytic signaling cascades that lead to melanization and coagulation reactions at injury sites and around foreign objects; (b) they utilize blood cells to either phagocytose or encapsulate invading microbes; and (c) they produce numerous antimicrobial peptides (for review, see Hoffmann and Reichhart 1997; Khush and Lemaitre 2000). Antimicrobial peptides are synthesized in the fat body (an analog of the mammalian liver) in response to systemic infections and in several epithelial tissues in response to local infections (Ferrandon et al. 1998; Tzou et al. 2000; Onfelt Tingvall et al. 2001). Seven distinct classes of peptides have been identified in *Drosophila*: Drosomycin, Metchnikowin, Diptericin, Drosocin, Cecropin, Defensin, and Attacin. Drosomycin is mainly active against

fungi; Metchnikowin shows both antifungal and antibacterial activities; the other peptides are mainly active against bacteria (for review, see Hoffmann and Reichhart 1997). Rel/Nuclear Factor (NF)- κ B-like transcription factors are the major regulators of antimicrobial peptide gene expression in *Drosophila*, and their activity is modulated by signaling pathways that are similar to the interleukin 1-receptor (IL1-R) in mammals (for review, see Engstrom 1999; Anderson 2000; Khush et al. 2001). These similarities indicate an evolutionary link between the control of antimicrobial peptide gene expression and the control of mammalian innate immune responses (Hoffmann et al. 1999; Aderem and Ulevitch 2000).

The Toll signaling pathway, which was first identified as a regulator of embryonic dorsal-ventral patterning, is one regulator of antimicrobial peptide gene expression (Lemaitre et al. 1996). Upon infection, the Spaetzle (Spz) protein is cleaved to generate a ligand for the Toll transmembrane receptor protein; Toll binding by Spz stimulates the degradation of the Inhibitory (I)- κ B homolog, Cactus, and the nuclear translocation of the Rel proteins Dorsal and Dorsal-like immunity factor (Dif). Dorsal and

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Dif appear to function redundantly to regulate antimicrobial peptide gene expression in larvae, but in adults, Dif is required for Toll-mediated antimicrobial peptide gene induction (Manfrulli et al. 1999; Meng et al. 1999; Rutschmann et al. 2000a). Loss-of-function mutations in genes of the Toll signaling cascade (*spz*, *Toll*, *pelle*, and *tube*) impair the induction of the antifungal peptide gene *Drosomycin*, and in flies carrying a gain-of-function *Toll* mutation that activates the Toll pathway, *Drosomycin* is constitutively expressed (Lemaitre et al. 1996). Toll-deficient flies resist bacterial infections but are highly susceptible to fungal infections, demonstrating that the immune responses regulated by the Toll pathway are required to resist fungal infection (Lemaitre et al. 1996).

The second pathway regulating antimicrobial peptide gene expression in flies was initially identified by a mutation in the *immune deficiency* (*imd*) gene that results in susceptibility to Gram-negative bacterial infection and an impairment of antibacterial peptide gene expression (Lemaitre et al. 1995). *imd* encodes a homolog of the mammalian Receptor Interacting Protein (RIP) (P. Georgel et al., in prep.), and molecular studies have isolated four additional factors that appear to define the Imd pathway: Relish, a third *Drosophila* Rel protein (Hedengren et al. 1999); two members of a *Drosophila* I κ B kinase (IKK) complex, that is, the kinase DmIKK β and a structural component DmIKK γ (Kim et al. 2000; Rutschmann et al. 2000b; Silverman et al. 2000; Lu et al. 2001) and Dredd, a caspase (Elrod-Erickson et al. 2000; Leulier et al. 2000). Relish is a homolog of the mammalian P100 and P105 compound Rel proteins that contain both Rel domains and inhibitory ankyrin domains (Hedengren et al. 1999). A receptor for the Imd pathway has not been identified, but infection triggers Relish cleavage and nuclear translocation of the Rel domain (Stöven et al. 2000). Relish cleavage requires DmIKK β activity, indicating that, like the mammalian IKK complex that functions in the IL1-R and TNF-R pathways, the *Drosophila* IKK complex regulates Rel protein activity (Silverman et al. 2000). In contrast to P100 and P105 processing, however, Relish cleavage is not blocked by proteasome inhibitors but does require a functional *Dredd* gene, suggesting that Dredd may cleave Relish directly after infection (Stöven et al. 2000). Like *imd*, mutations in *DmIKK β* , *DmIKK γ* , *Dredd*, and *Relish* affect antibacterial peptide gene expression after infection and induce susceptibility to Gram-negative bacterial infections (Hedengren et al. 1999; Leulier et al. 2000; Rutschmann et al. 2000b; Lu et al. 2001). However, mutations in these genes do not induce susceptibility to fungal infections, demonstrating that the immune responses regulated by the Imd pathway are required to resist Gram-negative bacterial but not fungal infections (Leulier et al. 2000; Rutschmann et al. 2000b).

Some significant conclusions of recent studies on the regulation of *Drosophila* antimicrobial peptide gene expression are that the Toll and Imd pathways do not share any components and that each pathway regulates specific Rel proteins (Leulier et al. 2000; Rutschmann et al. 2000b; Khush et al. 2001; Lu et al. 2001). The only evi-

dence of interactions between the two pathways is the observation that both pathways are required to fully induce some of the antimicrobial peptide genes (Lemaitre et al. 1996), suggesting that these genes respond to combinations of Rel proteins controlled by the two pathways. The influence of each pathway on the expression of each antimicrobial peptide gene is apparent in flies carrying mutations that affect either the Toll or the Imd pathway: *Drosomycin* is mainly controlled by the Toll pathway; *Diptericin* and *Drosocin* can be fully activated by the Imd pathway; and full *Metchnikowin*, *Defensin*, *Cecropin A* and *Attacin* activation requires both pathways (Lemaitre et al. 1996; Levashina et al. 1998). None of the antimicrobial peptide genes are induced in *imd*; *Toll* double mutant flies, demonstrating that Imd and Toll are two essential pathways that regulate antimicrobial gene expression pathways (Lemaitre et al. 1996).

Despite our increased understanding of the regulation of antimicrobial peptide gene expression in flies, various intermediates in the Toll and Imd pathways remain uncharacterized: For example, the kinase that targets Cactus for degradation in the Toll pathway and the receptor-adaptor complex that regulates the Imd pathway have not been identified. Following the observations that null mutations affecting the Imd pathway are not required for viability (Hedengren et al. 1999; Leulier et al. 2000; Rutschmann et al. 2000b; Lu et al. 2001), we initiated a search for additional members of the Imd pathway by screening for nonlethal mutations that induce susceptibility to Gram-negative bacterial infection in adult flies. We now report that null mutations in the *Drosophila transforming growth factor activated kinase 1* gene (*dTAK1*) encoding the *Drosophila* homolog of the mammalian mitogen-activated protein kinase kinase kinase (MAPKKK) TAK1 (Takatsu et al. 2000) induce high susceptibility to Gram-negative bacterial infection and block antibacterial peptide gene expression. Our results indicate that *dTAK1* codes for a new component of the Imd pathway.

Results

D10 is required to resist Gram-negative bacterial infection

To identify *Drosophila* genes that mediate defense reactions to bacterial infection, we tested ~2500 lines carrying ethyl methanesulfonate (EMS)-induced mutations on the X chromosome for susceptibility to bacterial infection: we pricked male adult flies with a needle dipped into a pellet of the Gram-negative bacterial species *Erwinia carotovora carotovora 15* (*E. carotovora 15*) (Basset et al. 2000) and screened for mutants that failed to survive infection. Using this assay, we isolated nine recessive, homozygous viable mutations that render flies highly susceptible to *E. carotovora 15* infection: Less than 10% of the mutated flies survived 48 h postinfection, whereas more than 90% of the wild-type flies survived (data not shown). These nine mutations fall into two complementation groups: *B118*, which represents five of the mutations, and *D10*, which represents the

other four mutations. The *B118* group corresponds to the caspase encoding gene *Dredd* that we described recently (Leulier et al. 2000), and in the present report we characterize *D10*, the second gene isolated in our screen.

To compare the *D10* phenotype with the phenotypes generated by mutations in other genes that regulate *Drosophila* immune responses, we first assayed the susceptibility of *D10* and other mutant lines to infection by four microorganisms: we pricked flies with the Gram-negative bacteria *Escherichia coli* (*E. coli*), the Gram-positive bacteria *Micrococcus luteus* (*M. luteus*), or the fungus *Aspergillus fumigatus*, and we naturally infected flies with the entomopathogenic fungus *Beauveria bassiana* (*B. bassiana*) (Fig. 1). The *D10* phenotype is similar to the *imd* and *Relish* phenotypes; flies carrying the *D10*, *imd*, and *Relish* mutations are susceptible to Gram-negative bacterial infection and resistant to Gram-positive bacterial and fungal infections, although *D10* flies, like *imd* flies, exhibit slightly lower susceptibility to Gram-negative bacterial infection compared to *Relish* mutants. In contrast, mutations in the *spz* gene render flies susceptible to fungal infections, and only flies carrying mutations in both *spz* and *imd* are susceptible to Gram-positive bacterial infection. This survival analysis demonstrates that the *D10* gene product, like *Imd* and

Relish, is required to resist Gram-negative bacterial infection.

D10 regulates antibacterial peptide gene expression

The Toll pathway is required for the full induction of the antifungal peptide genes and a subset of the antibacterial peptide genes. Mutations that block the Toll pathway reduce the expression of these genes; conversely, mutations that block the *Imd* pathway reduce the expression of genes with antibacterial activity (for review, see Engstrom 1999; Khush et al. 2001). To determine how the *D10* mutation affects antimicrobial peptide gene expression, we monitored the levels of *Diptericin*, *Cecropin A*, *Defensin*, and *Attacin*, which encode antibacterial peptides, *Drosomycin*, which encodes an antifungal peptide, and *Metchnikowin*, which encodes a peptide with both antibacterial and antifungal activity (Hoffmann and Reichhart 1997), in flies homozygous for two *D10* alleles. In addition, we compared the *D10* phenotype with all of the previously identified mutations affecting the *Imd* pathway and with a *spz* mutation that blocks the Toll pathway.

Pricking adult flies with a mixture of Gram-positive and Gram-negative bacteria activates the expression of

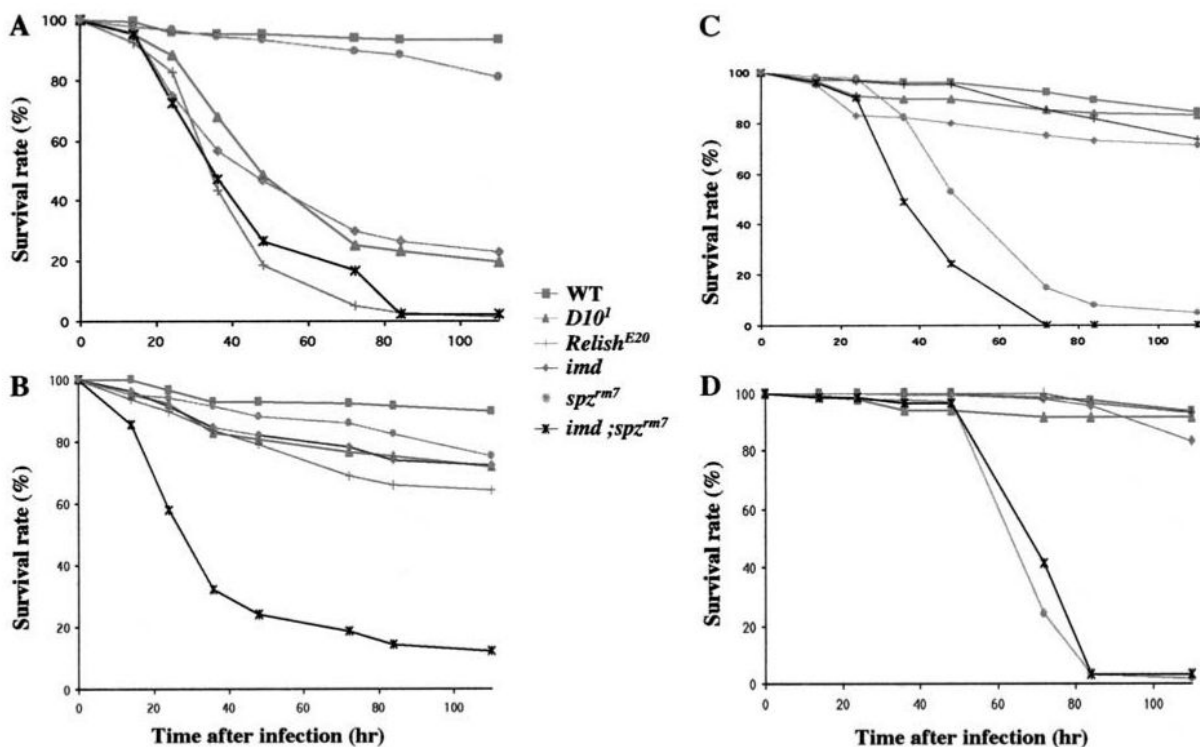


Figure 1. *D10* is required to resist Gram-negative bacterial infection. The survival rates of wild-type [Canton^S, ■], *imd* (◆), *spz*^{rm7} (●), *D10*¹ (▲), *Relish*^{E20} (+) and *imd; spz*^{rm7} (⊗) flies after different types of infection are presented. From 100 to 200 adults, aged 2–4 d, were pricked with a needle previously dipped into one of the following: *E. coli* (A); *M. luteus* (B); *Aspergillus fumigatus* (C), or naturally infected by *B. bassiana* (D). The infected flies were incubated at 29°C and transferred to fresh vials every 3 d. All of the different mutants flies tested exhibited more than 80% survival 100 h after challenge by a clean injury (data not shown). Mutations in *D10*, *Relish*, and *imd* render adult flies highly susceptible to *E. coli* infection (A). Only flies carrying both the *imd* and *spz* mutation are sensitive to *M. luteus* infection (B). The *spz* gene is required to resist fungal infections (C and D).

all the antimicrobial peptide genes; in the *D10* mutants, however, mixed Gram-negative/Gram-positive infections induce significant levels of only *Drosomycin* and *Metchnikowin* (Fig. 2A,B). Quantitative measurements of three independent RNA blot experiments show that in *D10* flies, *Drosomycin* is induced to wild-type levels, *Metchnikowin* is induced to 70% of wild-type levels, *Cecropin A*, *Defensin*, and *Attacin* are induced to <25% of wild-type levels, and *Diptericin* is induced to <5% of wild-type levels (Fig. 2B). This pattern of antimicrobial peptide gene expression in the *D10* mutants is similar to the patterns displayed in mutants of the Imd pathway,

although the *D10* mutations, like *imd*, have slightly weaker effects on antimicrobial peptide gene expression compared to the *Dredd*, *DmIKK β* , *DmIKK γ* , and *Relish* mutations (Fig. 2A) [Lemaitre et al. 1995; Hedengren et al. 1999; Leulier et al. 2000; Rutschmann et al. 2000b; Lu et al. 2001]. This weaker phenotype in *D10* and *imd* flies correlates well with their lower susceptibility to *E. coli* infection (Fig. 1).

D10 does not function in the Toll pathway

In contrast to the infections induced by pricking flies with mixtures of Gram-negative and Gram-positive bacteria, natural infections by the fungus *B. bassiana* selectively induce the expression of *Drosomycin* and *Metchnikowin* via the Toll pathway (Lemaitre et al. 1997). To determine whether *D10* plays a role in the Toll pathway, we compared *Drosomycin* expression in different mutant backgrounds after fungal infection. We found that in contrast to mutations in *spz*, mutations in *D10* and components of the Imd pathway do not block *Drosomycin* induction (Fig. 3A); the variable *Drosomycin* levels in the different Imd pathway mutants are probably due to differing efficiencies of natural infection. Furthermore, mutations in *D10* and *Dredd* do not block the constitutive *Drosomycin* expression induced by the dominant, gain-of-function *Toll^{10B}* mutation (Lemaitre et al. 1996) (Fig. 3B). The Toll and Imd pathways are the two major regulators of antimicrobial peptide gene expression in *Drosophila*, and flies carrying mutations in both the Toll and Imd pathways do not express any antimicrobial peptide genes (Lemaitre et al. 1996). Similarly, flies carrying *spz* in combination with either *D10* or *Dredd* mutations fail to express any of the antimicrobial peptide genes after a mixed bacterial infection (Fig. 3C). We noted only a residual expression of the *Drosomycin* gene in the double mutant. The *D10* phenotypes that we have characterized, that is, susceptibility to Gram-negative bacterial infection, reduced induction of the antimicrobial peptide genes, absence of detectable function in the Toll pathway, and a complete block of antimicrobial peptide gene expression in combination with a *spz* mutation, are identical to the phenotypes generated by mutations in members of the Imd pathway, suggesting that *D10* encodes a novel component of the Imd pathway.

D10 encodes the MAPKKK *dTAK1*

Using susceptibility to Gram-negative bacterial infection as an assay, we first mapped *D10* by recombination to a region of the X chromosome distal to *forked* (data not shown). Deficiencies and additional genetic markers enabled us to further localize *D10* to a portion of the cytogenetic interval 19E1 that, based on genomic annotation data (Consortium 1998), contains ~25 genes (Fig. 4A). One of the 25 genes in this region is *dTAK1*, which encodes a homolog of the mammalian *TAK1* gene product (Takatsu et al. 2000). In mammals, *TAK1* is implicated in the regulation of NF- κ B as a component of the

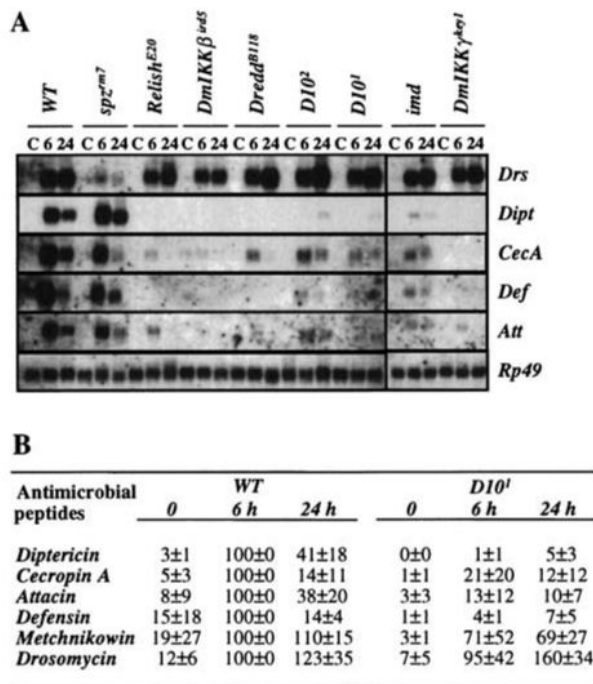


Figure 2. *D10* regulates the expression of antibacterial peptide genes in adults. (A) A time course of antimicrobial gene expression in different mutant adults infected with a mixture of *E. coli* and *M. luteus* shows that *D10*, *Dredd*, *Relish*, *DmIKK β* , *DmIKK γ* , and *imd* predominantly control the expression of the antibacterial genes (*Diptericin*, *Cecropin A*, *Attacin*, and *Defensin*). The Northern blot was performed with total RNA extracted from wild-type, *D10¹*, *D10²*, *Dredd^{B118}*, *imd*, *Relish^{E20}*, *DmIKK β ^{td5}*, *DmIKK γ ^{key1}* and *spz^{tm7}* mutant adults at different time intervals after infection (indicated in hrs). After infection, flies were incubated at 25°C. The blot was successively hybridized with the following cDNA probes: *Diptericin* (*Dipt*), *Cecropin A* (*Cec A*), *Attacin* (*Att*), *Defensin* (*Def*), *Drosomycin* (*Drs*) and *Rp49*. (C) Uninfected control flies. (B) The quantification of antimicrobial peptide gene expression in wild-type and *D10¹* mutant adults collected 6 h and 24 h after infection with a mixture of *E. coli* and *M. luteus*, expressed as the percentage of the induction seen in wild-type flies 6 h after infection. The signals from three independent Northern blot experiments were quantified with a Bio-Imager system, and the levels of immune gene expression were normalized with the corresponding value of the *Rp49* signal. The average and standard deviation are shown.

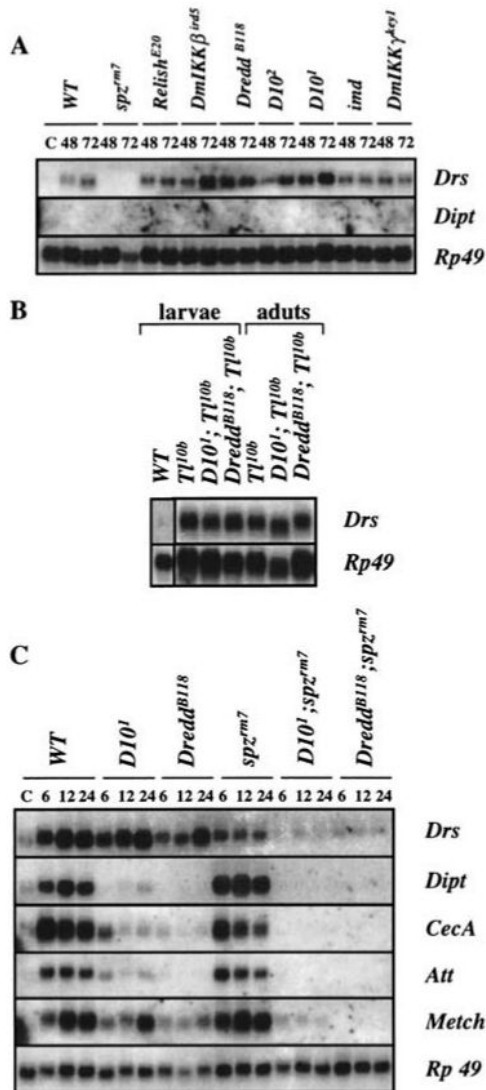


Figure 3. *D10* mutants do not interfere with the Toll-mediated induction of *Drosomycin* expression. (A) Antimicrobial gene expression patterns in different mutant adults collected either 48 h or 72 h after natural infection by the entomopathogenic fungi *B. bassiana* shows that natural infection by *B. bassiana* induces a selective expression of *Drosomycin* that is dependent on *spz* but not on *D10*, *Dredd*, *Relish*, *DmIKK β* , *DmIKK γ* , and *imd*, indicating that the Toll-mediated expression of the *Drosomycin* gene by this fungus is not altered by mutations affecting the Imd pathway. The Northern blot was performed as described in Fig. 2. Flies naturally infected were incubated at 29°C. The blot was successively hybridized with the following cDNA probes: *Diptericin* (*Dipt*), *Drosomycin* (*Drs*), and *Rp49*. (C) Uninfected control flies. (B) The high level of *Drosomycin* gene expression in uninfected *Tl10b*, *D10¹*, *Tl10b*, and *Dredd^{B118}*, *Tl10b* mutants demonstrates that *D10* and *Dredd* are not required for the *Tl10b*-driven constitutive expression of *Drosomycin* in larvae and adults. (C) *D10¹*, *spz^{rm7}* and *Dredd^{B118}*, *spz^{rm7}* double mutants fail to express any antimicrobial peptide gene after mixed Gram-negative/Gram-positive bacterial infection, indicating that another pathway cannot substitute for the Toll and Imd pathways to induce antimicrobial peptide gene expression.

IL-1 signaling pathway, where it is thought to function upstream of the IKK complex (Sakurai et al. 1998; Ninomiya-Tsuji et al. 1999; Sakurai et al. 1999; Irie et al. 2000). Based on the established roles of an IKK complex and the NF- κ B-like transactivator Relish in the Imd pathway, we selected *dTAK1* as a candidate for *D10*.

We performed several different experiments to determine whether the *D10* alleles correspond to mutations in *dTAK1*. First, we tested the ability of a 15 Kb genomic fragment that contains *dTAK1* and annotation *CG1812* (Fig. 4B) to rescue the immune response deficiency in *D10* flies. Figures 4C and D show that *D10* adults carrying the *dTAK1* transgene (*P[dTAK1⁺]*) both express *Diptericin* and resist Gram-negative bacterial infection at levels comparable to wild-type flies, demonstrating that this genomic fragment rescues the *D10* phenotypes. We also determined that overexpression of the *dTAK1* cDNA using the UAS/GAL4 system partially rescues *Diptericin* expression in the *D10¹* mutant (Fig. 6A, see below). We next sequenced the three Kb of *dTAK1* genomic coding sequence in our original y,w strain and in each of the four homozygous *D10* lines. The four *D10* alleles all contain mutations within the *dTAK1* kinase domain, which led us to rename the *D10* alleles *dTAK1¹* to *dTAK1⁴*: *dTAK1¹* and *dTAK1⁴* were generated by missense mutations in conserved residues, *dTAK1²* was generated by a point mutation that creates a stop codon, and *dTAK1³* contains a deletion of 31 base pairs that also results in a premature stop codon (Fig. 5A,B). All four *dTAK1* alleles inhibit *Diptericin* induction by Gram-negative bacterial infection to the same degree (Fig. 4C), and this inhibition is not enhanced in flies heterozygous for each allele and a deficiency spanning *dTAK1* (data not shown). In addition, flies homozygous for the four alleles are equally susceptible to Gram-negative bacterial infection (data not shown). The apparent null phenotype manifested by the four *dTAK1* alleles indicates that the *dTAK1* kinase domain is essential for *dTAK1* function in the Imd pathway. This observation is supported by results from our experiments with a kinase dead form of *dTAK1*, *dTAK1-K46R*, which was previously reported to act as a dominant negative inhibitor of *dTAK1* (Takatsu et al. 2000). *dTAK1-K46R* expression driven by the UAS/GAL4 system (Brand and Perrimon 1993) blocks *Diptericin* expression after mixed bacterial infection, confirming that *dTAK1* is required for the *Drosophila* antibacterial immune response (Fig. 4C). The results of our rescue experiments, our sequencing data, and the dominant negative *dTAK1* mutant phenotypes together demonstrate that *D10* encodes the MAPKKK *dTAK1*.

dTAK1 functions upstream and *Dredd* functions downstream of the *Drosophila* IKK complex

The loss-of-function *dTAK1* mutations that we isolated display immune response phenotypes that are very similar to the phenotypes generated by mutations in *imd*, *DmIKK β* , *DmIKK γ* , *Dredd*, and *Relish*, suggesting that these genes function together in the Imd pathway. Pre-

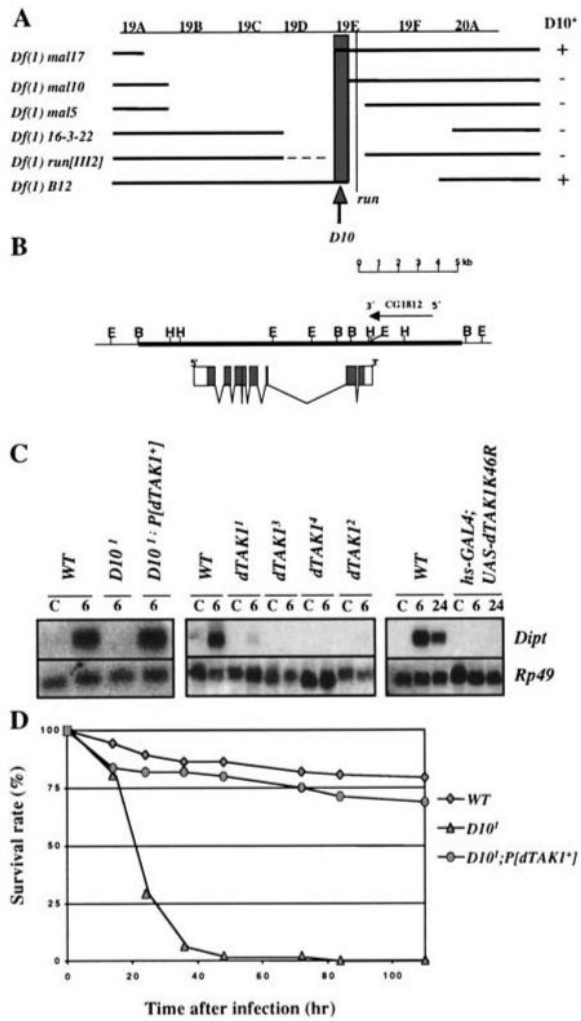


Figure 4. *D10* encodes *dTAK1*. (A) Mapping the *D10* region with deficiencies. A genetic map of the 19C-20A region of the X chromosome with the positions of the *D10* gene and *run*. Deficiencies are indicated with their approximate breakpoints. The deficiencies that complement *D10* (+) or do not complement *D10* (-) are indicated on the right. Resistance to *E. carotovora* 15 infection was used to assay for the presence of the *D10* gene. According to these complementation tests, *D10* maps to region 19E1 (as indicated by the shaded box). (B) A genomic map of the *dTAK1* locus showing the exon-intron organization of the gene and the 15 kb genomic DNA contained in the *P[dTAK1⁺]* transgene. Annotation *CG1812* is indicated. (C) Northern blot analysis of total RNA extracted from adult flies infected with a mixture of Gram-positive (*M. luteus*) and Gram-negative (*E. coli*) bacteria shows that *Diptericin* expression is restored in *D10⁺*; *P[dTAK1⁺]* flies (left panel). The four alleles of *dTAK1* (1-4) completely block *Diptericin* induction after mixed bacterial infection (center panel). The overexpression of a dominant negative form of *dTAK1* also blocks *Diptericin* induction in adult flies carrying both the *hs-GAL4* and *UAS-dTAK1 K46R* transgenes collected 6 h and 24 h after bacterial challenge. A 1 h heat shock (37°C) was performed just before infecting the flies. (C) Uninfected control flies. (D) *D10* flies carrying the *P[dTAK1⁺]* transgene resist *E. carotovora* 15 infection at levels comparable to wild-type flies.

vious studies indicated that DmIKK γ and DmIKK β directly regulate Relish activity (Silverman et al. 2000); however, the positions of Imd, *dTAK1*, and Dredd in the Imd pathway were not determined. Double mutant analysis combining a *Toll* gain-of-function mutation that constitutively activates the Toll pathway and loss-of-function mutations in other genes was a powerful tool for establishing the order of action of the genes encoding components of the Toll pathway (for review, see Belvin and Anderson 1996). Unfortunately, none of the mutations affecting the Imd pathway produce constitutive antibacterial immune responses; consequently, to avail of a genetic approach for ordering the Imd pathway, we over-expressed both *Dredd* and *dTAK1* via the UAS/GAL4 system. In lines carrying a heat shock (*hs*)-GAL4 driver and either the *Dredd* or *dTAK1* cDNAs under the control of a UAS promoter, heat shock induces *Diptericin* expression to about 15–20% of the level observed in adults 6 h after bacterial infection (Fig. 6A). This result indicates that the overexpression of these two genes is

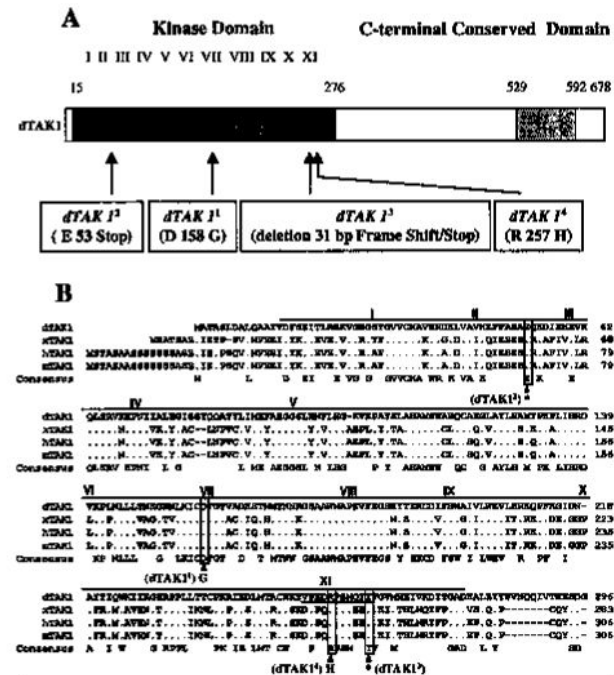


Figure 5. The *dTAK1* mutations affect the *dTAK1* kinase domain. (A) The four EMS-induced alleles of *dTAK1* each contain a single point mutation, or small deletion [*dTAK1³*] that generate the indicated changes in the kinase region of the *dTAK1* protein. (B) The amino acid sequence of the *dTAK1* kinase domain is compared with that of *Xenopus* (x) TAK1, one of three different forms of human (h) TAK1, and mouse (m) TAK1 (Takatsu et al. 2000). The exact positions of the four mutations in *dTAK1* are indicated. The *dTAK1¹* and *dTAK1⁴* mutations induce amino acid substitutions in conserved residues of kinase domains 7 and 11, respectively. The *dTAK1²* and *dTAK1³* mutations generate stop codons (*) in the beginning or the end of the kinase domain. The stop codon in *dTAK1³* is generated by a deletion of 31 bp that produces a frameshift (the deleted codons are underlined).

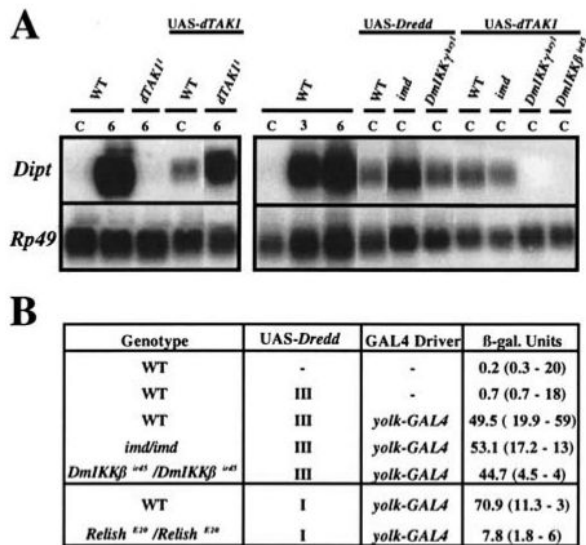


Figure 6. A genetic analysis of the Imd pathway. (A) Overexpression of *UAS-dTAK1* and *UAS-Dredd* [isoform δ^*] using the *UAS/GAL4* system was able to partially or completely rescue the *dTAK1* and *Dredd* mutations, respectively (*dTAK1*, left panel; *Dredd*, data not shown). Overexpression of these two constructs with an *hs-GAL4* driver induces a weak but consistent expression of the *Diptericin* gene after heat shock in absence of infection (right panel). By classic genetic techniques, the *UAS* and *GAL4* constructs were placed in the *imd*, *DmIKK β* , and *DmIKK γ* genetic backgrounds. The right panel shows that the *Diptericin* expression induced by overexpressing *dTAK1* is blocked by the *DmIKK β* and *DmIKK γ* mutations but not by the *imd* mutation. In contrast, *Dredd*-mediated expression of *Diptericin* is not blocked in the *DmIKK γ* and *imd* mutant backgrounds. Total RNA for Northern Blot analysis was extracted from adult flies collected 24 h after a 1 h heat shock (37°C) that carry one copy of either a *UAS-dTAK1* transgene (II chromosome) or a *UAS-Dredd* transgene (III chromosome) and one copy of the *hs-GAL4* transgene (III chromosome). In the case of *Dredd*, we use a recombinant line carrying both *UAS-Dredd* and *hs-GAL4* on the same chromosome. (B) Overexpression of *UAS-Dredd* by the *yolk-GAL4* driver induced a high level of *Diptericin-lacZ* activity in absence of challenge that is dependent on *Relish* but not the *imd* or the *DmIKK β* genes. Unchallenged female adult flies were generated that carry one copy of a *Diptericin-lacZ* (X chromosome) reporter gene in combination with one copy of a *UAS-Dredd* transgene (X or III chromosomes) and one copy of the *yolk-GAL4* driver transgene (III chromosome) in wild-type, *imd*, *DmIKK β* , and *Relish* mutant backgrounds. The first column shows the genotype, and the second and third columns indicate the presence or absence (-) of the *UAS-Dredd* and *yolk-GAL4* driver, respectively. The last column shows the level of *Diptericin-lacZ* expression monitored by LacZ titration (Manfrulli et al. 1999). Means for *Diptericin-lacZ* expression are shown with standard deviation, and the number of repeats are given in brackets. The *yolk-GAL4* driver expresses GAL4 specifically in the female adult fat body. The level of *Diptericin-lacZ* in 6 h-challenged females was 21 (4.6; 26).

sufficient to activate the antibacterial pathway in the absence of infection. By using this *UAS/GAL4* system to overexpress *dTAK1* and *Dredd* in various mutant back-

grounds, we tested the epistatic relationships between *dTAK1* and *Dredd*, and the other genes of the Imd pathway. Figure 6A shows that mutations in *DmIKK β* and *DmIKK γ* , but not *imd*, block *Diptericin* induction by *dTAK1* overexpression, indicating that *dTAK1* functions downstream of Imd and upstream of the IKK complex. However, the *Diptericin* expression induced by *Dredd* overexpression was not affected by mutations in *imd* or *DmIKK γ* . For genetic reasons, we were unable to use Northern blot analysis to test the effect of mutations in *Relish* and *DmIKK β* , which are on the third chromosome, on the *UAS-Dredd*-induced *Diptericin* expression. Therefore, we overexpressed the *UAS-Dredd* transgene through a female, adult fat body driver (*yolk-GAL4*) and monitored *Diptericin* expression with a *Diptericin-LacZ* reporter gene (Fig. 6B). LacZ titration assays demonstrate that β -galactosidase activity is induced in lines overexpressing *UAS-Dredd* in the absence of infection. The *Dredd*-mediated *Diptericin-LacZ* induction was strongly reduced in *Relish* but not in *DmIKK β* mutants, confirming our Northern blot results showing that *Dredd* functions downstream of the IKK complex and demonstrating that *Dredd* regulates *Diptericin* expression through *Relish*.

Natural Gram-negative bacterial infection selectively activates the Imd pathway

Natural fungal infections highlight the ability of *Drosophila* to discriminate between pathogens and activate specific immune response pathways that lead to adapted immune responses (for review, see Khush and Lemaitre 2000). Although only the Imd pathway is required to resist Gram-negative bacterial infection, the Toll pathway is still activated to some degree in flies pricked with Gram-negative bacteria (Lemaitre et al. 1997). This suggests that injury (and associated contamination) contributes to a nonspecific immune response. We recently demonstrated that *E. carotovora 15* naturally infects the *Drosophila* larval gut and triggers both the local and systemic expression of antimicrobial peptide genes (Basset et al. 2000; Tzou et al. 2000). In contrast to infection by pricking, natural *E. carotovora 15* infection induces *Diptericin* expression more strongly than *Drosomycin* expression (Basset et al. 2000). To compare the contribution of the Toll and Imd pathways to antimicrobial peptide induction after natural *E. carotovora 15* infection, we quantified *Diptericin* and *Drosomycin* expression in larvae from various mutant lines after natural *E. carotovora 15* infections. Figure 7 shows that the *Diptericin* expression induced by *E. carotovora 15* natural infection is entirely dependent on the genes of the Imd pathway, confirming that *Diptericin* is exclusively regulated by the Imd pathway. Interestingly, the level of *Diptericin* induction was higher in *spz* mutants than in wild-type larvae. In addition, *Drosomycin* induction after *E. carotovora 15* natural infection of larvae was also dependent on the Imd pathway: mutations blocking the Imd pathway have stronger effects than the *spz* mutation on *Drosomycin* expression induced by natural infection. These

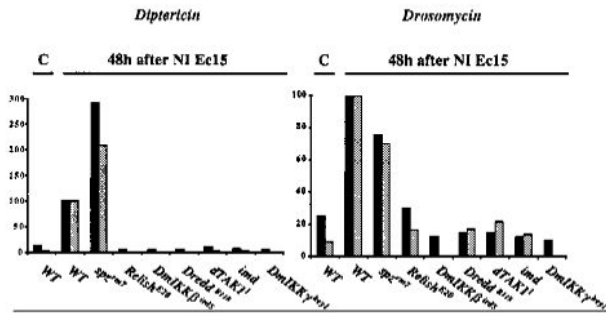


Figure 7. The Imd pathway regulates the expression of antimicrobial genes in larvae after infection by Gram-negative bacteria. The quantification of *Diptericin* and *Drosomycin* expression in wild-type and various mutant larvae collected 48 h after natural infection by *E. carotovora 15* is expressed as the percentage of the induction seen in wild-type larvae. The signals from two independent Northern blot experiments were quantified with a Bio-Imager system, and the levels of antimicrobial peptide gene expression were normalized with the corresponding value of the *Rp49* signal. Each bar represents the result of a single experiment. This results shows that *dTAK1*, *Dredd*, *Relish*, *DmIKK β* , *DmIKK γ* , and *imd* control the expression of the antibacterial genes *Diptericin* and *Drosomycin* after natural infection by *E. carotovora 15*. Larvae naturally infected by *E. carotovora 15* were incubated at 29°C.

data contrast somewhat with our previously published observation that the *imd* mutation did not block *Drosomycin* induction by *E. carotovora 15* infection (Basset et al. 2000). However, our first analysis of *Drosomycin* induction by *E. carotovora 15* was based on the qualitative analysis of a *Drosomycin-lacZ* reporter gene; we believe that the quantitative analysis of *Drosomycin* expression by Northern blot is a more accurate determination of *Drosomycin* expression patterns. In conclusion, our current results indicate that *Drosomycin* gene induction after natural Gram-negative bacterial infection is largely mediated by the Imd pathway.

Natural infections by *E. carotovora 15* also trigger the local expression of antimicrobial peptide genes in various epithelial tissues, and both *Diptericin* expression in the anterior midgut and *Drosomycin* expression in the trachea are dependent on the *imd* gene (Ferrandon et al. 1998; Tzou et al. 2000). By assaying the expression of *Diptericin-lacZ* and *Drosomycin-GFP* reporter genes in naturally infected *dTAK1* and *Dredd* mutant larvae, we now show that both genes are required for *Diptericin* and *Drosomycin* expression in epithelial tissues after natural *E. carotovora 15* infection (Fig. 8). These data confirm the predominant role of the Imd pathway in antimicrobial peptide regulation after natural *E. carotovora 15* infection and suggest that Gram-negative bacterial recognition in flies preferentially activates the Imd pathway.

Discussion

The *Drosophila* homolog of TAK1 regulates antibacterial defense

Mammalian TAK1 was initially identified by a complementation assay in yeast for its ability to substitute for

the MAPKKK Ste11p in the yeast MAPK pheromone pathway (Yamaguchi et al. 1995). TAK1 is a MAPKKK and contains a protein kinase domain in its N-terminal region that is ~30% identical to the catalytic domains of RAF1 and MEKK1. In mammals, TAK1 function has been extensively studied in transient transfection assays using cultured cells (for review, see Behrens 2000). These studies indicate that TAK1 can function in a signal transduction pathway that is triggered by the TGF β superfamily of ligands. In addition, the overexpression of *Xenopus* TAK1 and an upstream activator called TAB1 induce embryonic ventralization. Studies of dominant negative mutant forms of TAK1 place TAK1 activity downstream of the BMP2/4 receptors (Shibuya et al. 1998).

Besides TGF β signaling, TAK1 appears to be involved in various other signal transduction pathways. Cell culture experiments indicate that ceramide stimulates the TAK1 kinase activity and that ceramide-induced JNK/SAPK activation is blocked by a dominant negative TAK1 mutant (Shirakabe et al. 1997). Biochemical studies also show that MAPKK4 and MAPKK3/MAPKK6 can be phosphorylated by TAK1, suggesting that TAK1 can activate the JNK pathway and/or the P38 MAPK pathways (Moriguchi et al. 1996; Wang et al. 1997), and other experiments demonstrate interactions between TAK1 and the Wnt signaling pathway (Ishitani et al. 1999). Several studies have pointed toward a role for TAK1 in the IL1-R/NF- κ B signaling pathway (Sakurai et al. 1998; Ninomiya-Tsuji et al. 1999; Sakurai et al. 1999). Dominant negative TAK1 mutants prevent NF- κ B activation by IL1 or the TLRs (Ninomiya-Tsuji et al. 1999; Irie et al. 2000), and TAK1 appears to activate NF- κ B by stimulating the I κ B kinase complex (IKK) that induces I κ B degra-

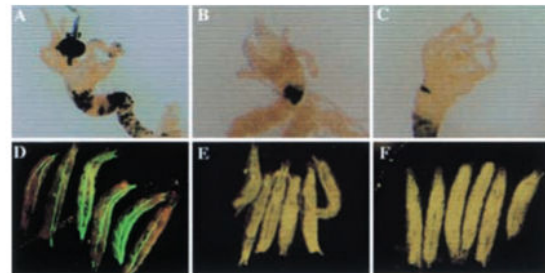


Figure 8. The Imd pathway regulates the local expression of *Diptericin* and *Drosomycin* in larvae after infection by Gram-negative bacteria. (A–C). The expression of the *Diptericin-lacZ* reporter gene in the midgut after natural infection by *E. carotovora 15* is blocked in *dTAK1*¹ and *Dredd*^{B118} mutants. Wild-type (A), *dTAK1*¹ (B), and *Dredd*^{B118} (C) mutant larvae carrying the *Diptericin-lacZ* reporter gene were collected 24 h after natural infection by *E. carotovora 15* at 29°C and stained for *lacZ* activity as described (Tzou et al. 2000). (D–F). The expression of the *Drosomycin-GFP* reporter gene in trachea after natural infection by *E. carotovora 15* is blocked in *dTAK1*¹ and *Dredd*^{B118} mutants. Wild-type (D), *dTAK1*¹ (E), and *Dredd*^{B118} (F) larvae carrying the *Drosomycin-GFP* reporter gene were collected 5 d after natural infection by *E. carotovora 15* at 18°C and directly observed under an epifluorescence microscope.

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dation by the proteasome (Ninomiya-Tsuji et al. 1999; Sakurai et al. 1999). Furthermore, TAK1 interacts with TRAF6, which is a downstream effector of the IL1 receptor, suggesting that TAK1 links TRAF6 to the NIK-IKK cascade in the IL1-R/TLR signaling pathways (Ninomiya-Tsuji et al. 1999).

A *Drosophila* homolog of TAK1, dTAK1, that possesses an extensively conserved NH2-terminal kinase and a partially conserved C-terminal domain was identified recently (Takatsu et al. 2000). Ectopic dTAK1 expression induces apoptosis, whereas overexpression of a dominant negative form of dTAK1 during development results in various cuticle defects, some of which are similar to those observed in mutants in JNK signaling. This preliminary analysis suggested a role for dTAK1 in the regulation of apoptosis and JNK signaling in flies (Takatsu et al. 2000; Mihaly et al. 2001). In the present study, we isolated four null mutations in dTAK1. Each of these mutations affects the kinase domain of dTAK1, generating either a premature stop codon or an amino acid substitution. Flies deficient for dTAK1 are highly susceptible to Gram-negative bacterial infection and fail to express antibacterial peptide-encoding genes after infection. This dTAK1 phenotype is identical to the phenotype induced by mutations affecting components of the Imd pathway, suggesting that dTAK1 is a new component of the Imd pathway. dTAK1 does not, however, function in the Toll pathway that mediates dorsal-ventral patterning and antifungal immune responses in flies; the dTAK1 mutations do not affect the Toll-mediated *Drosomycin* induction by fungal infection or the constitutive *Drosomycin* expression in *Toll* gain-of-function mutants, and the dTAK1 mutants are not susceptible to fungal infection. Surprisingly, our results indicate that, in contrast to TGF β - or JNK signaling-defective mutants, dTAK1 flies are viable and do not display obvious developmental defects. The developmental phenotypes generated by overexpressing a dominant negative form of TAK1 (Takatsu et al. 2000; Mihaly et al. 2001) were not observed in the dTAK1-deficient mutants, although we have shown that the dominant negative dTAK1 mutant does phenocopy the dTAK1 mutant immune phenotype. Our results demonstrate that dTAK1 is either not involved in JNK and TGF β signaling in *Drosophila* or that its function in these pathways is redundant with another MAPKKK.

Ordering the Imd pathway

Overexpressing dTAK1 or *Dredd* via the UAS/GAL4 system leads to weak but consistent *Diptericin* expression in the absence of infection, indicating that overexpressing these two genes is sufficient to turn on the antibacterial pathway. The constitutive *Diptericin* expression in the dTAK1 and *Dredd* overexpression lines also allowed us to analyze the epistatic relationship between dTAK1, *Dredd*, and the other recently identified regulators of antibacterial defense, and, in agreement with their similar mutant phenotypes, our results confirm that Imd, dTAK1, the DmIKK complex, and *Dredd* func-

tion in a pathway that culminates in Relish activation (Fig. 9). Our data suggest a model in which Imd functions upstream of dTAK1 and is the most upstream component currently identified in the Imd pathway. The *imd* gene was recently cloned and encodes a death domain-containing protein with homology to the mammalian RIP (P. Georgel et al., in prep.). In mammals, RIP appears to function in an adaptor complex associated with the tumor necrosis factor (TNF) receptor, and our genetic placement of *imd* suggests that IMD has a conserved function in flies as part of a receptor-adaptor complex that responds to Gram-negative bacterial infection (Fig. 9).

Our epistatic analysis also indicates that dTAK1 functions upstream of the DmIKK complex. In mammals, TAK1 functions upstream of the IKK complex, and it is not yet clear whether TAK1 directly activates the IKK

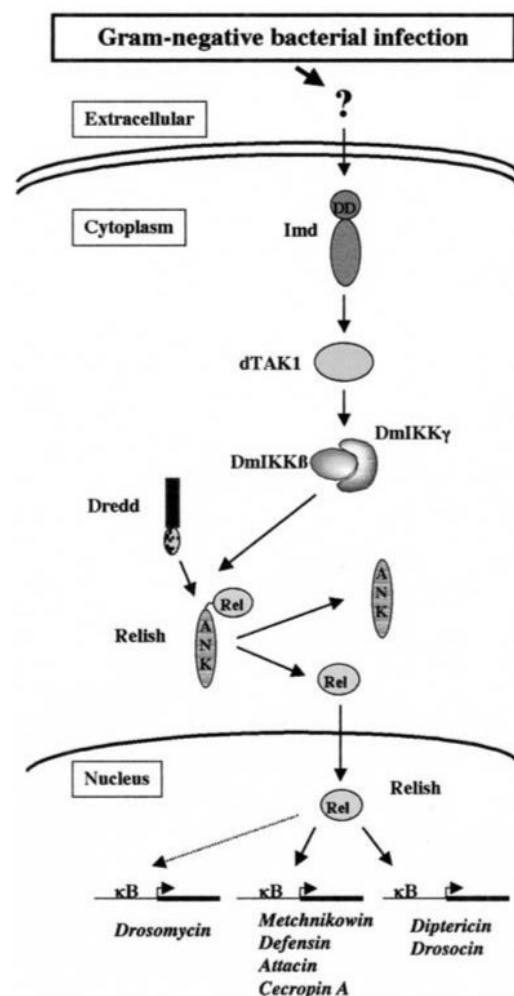


Figure 9. The Imd pathway. A model of the Imd pathway that is based on our epistatic studies shows that dTAK1 functions upstream and *Dredd* functions downstream of the DmIKK complex. Previous studies have shown that Relish is activated by the DmIKK complex (Silverman et al. 2000). See Discussion for additional details.

complex (Sakurai et al. 1999) or whether TAK1 functions through NIK, another MAPKKK that is involved in NF- κ B regulation (Ninomiya-Tsuji et al. 1999). A strong NIK homolog is not apparent in the *Drosophila* genome (Khush and Lemaitre 2000), suggesting that dTAK1 may directly activate the DmIKK complex after infection. *dTAK1* flies have slightly weaker phenotypes than *DmIKK β* , *DmIKK γ* , *Dredd*, and *Relish* mutants, and this may correlate with the function of dTAK1 at an early stage of the antibacterial cascade where multiple inputs, some of which bypass dTAK1, regulate the DmIKK complex.

Dredd encodes a caspase with a large prodomain and is related to mammalian Caspase 8 (Rodriguez et al. 1999). Our genetic analysis indicates that *Dredd* functions downstream of the DmIKK complex and induces *Diptericin* expression through *Relish*, which confirms previous observations by Stöven et al. (2000), who showed that after infection *Relish* is rapidly cleaved by a proteasome-independent mechanism and that this cleavage is blocked in *Dredd* mutants. There is increasing evidence that caspases are involved in physiological processes other than apoptosis, and *Dredd* function downstream of the DmIKK complex suggests that *Dredd* participates directly, or indirectly via an effector caspase, in *Relish* cleavage. In mammals, apical caspases are activated by the processing of a prodomain, and a future issue to address is whether *Dredd* is constitutively active or is regulated by the DmIKK complex or another upstream component of the Imd pathway.

Independent activation of the Toll and Imd pathways

A current view of the antimicrobial response in *Drosophila* is that the induction of the antimicrobial peptides are controlled by two distinct pathways, Toll and Imd, that each regulate specific rel/NF- κ B proteins (Leulier et al. 2000; Rutschmann et al. 2000b; Khush et al. 2001). These pathways are the two main immunoresponsive signaling cascades, as illustrated by our observation that flies carrying a mutation in both pathways fail to express any antimicrobial peptide genes and are highly susceptible to infection by all types of microorganisms. The ability to differentiate between microbes and activate each of these pathways independently, in response to different types of infection, appears to be one mechanism for mounting adapted immune responses. For example, the specific induction of *Drosomycin* after natural infection by the entomopathogenic fungus *B. bassiana* is due to the selective activation of the Toll pathway since, as indicated by our present results, this induction is not affected by mutations that block the Imd pathway. Natural infection by the Gram-negative bacterial species *E. carotovora 15* leads to a predominant expression of antibacterial rather than antifungal peptide genes, and reveals the ability of *Drosophila* to mount adapted innate immune responses to Gram-negative bacterial infection (Basset et al. 2000). Our present findings indicate that antimicrobial peptide gene expression after natural

E. carotovora 15 infections is largely mediated through the Imd pathway, and that both *Diptericin* and *Drosomycin* expression after *E. carotovora 15* infection are impaired in mutants affecting components of the Imd pathway. The high level of *Diptericin* expression and the low level of *Drosomycin* expression observed in this context probably reflect the differential responses of these two target genes to the selective activation of the Imd pathway. In the trachea, a major site of infection in insects, natural infection by *E. carotovora 15* triggers *Drosomycin* gene expression in an Imd-dependent manner (Tzou et al. 2000; present finding). This illustrates the ability of the *Drosomycin* gene to be weakly turned on in response to Gram-negative bacterial infection by the Imd pathway, independently of any Toll input.

Interactions between the Toll and Imd pathways

Except for *Diptericin* and *Drosocin*, all of the antimicrobial peptide genes are regulated by inputs from both the Toll and Imd pathways; some, like *Defensin*, require the concomitant activation of both pathways, while others, such as *Drosomycin* and *Metchnikowin*, can be induced independently by each of these pathways (Lemaitre et al. 1996; Levashina et al. 1998). One hypothesis is that the differential regulation of antimicrobial peptide genes reflects their sensitivity to specific combinations of the Rel proteins that are the end targets of the Toll and Imd pathways (Han and Ip 1999). For example, *Diptericin* induction after infection is fully dependent on *Relish* and is not altered in mutants deficient for both *Dif* and *Dorsal* activity (Hedengren et al. 1999; Manfrulli et al. 1999; Meng et al. 1999). In agreement, *Diptericin* gene expression is almost completely abolished in mutants deficient in Imd signaling. Interestingly, we observed that the level of *Diptericin* gene expression is higher in *spz* mutant larvae than in wild-type larvae after natural Gram-negative bacterial infection (Fig. 7), and we observed a similar response after mixed Gram-negative/Gram-positive bacterial infection in adult flies (Fig. 2A). These observations are consistent with our previous studies of *Diptericin* expression in *spz* mutants, although those results were not highlighted earlier (Fig. 2 in Lemaitre et al. 1996; Fig. 2A in Leulier et al. 2000). The effect of the *spz* mutation on *Diptericin* induction points to a negative effect of the Toll pathway on this gene. Our hypothesis is that *Relish* homodimers are the most potent activators of *Diptericin*, and that by blocking *Dif* and *Dorsal* activation, mutations in the Toll pathway may increase the formation of *Relish* homodimers versus *Relish*-*Dif* or *Relish*-*Dorsal* heterodimers. Together these observations indicate how the differential activation of these two pathways leads in a subtle way to the preferential expression of specific target genes.

Concluding remarks

We describe here the identification of null mutations in the *Drosophila* homolog of TAK1. Our genetic analysis

shows that *dTAK1*, like the other components of the Imd pathway, is required for the induction of antibacterial peptide gene expression, and *dTAK1*'s role in the *Drosophila* immune response is highlighted by the strong susceptibility of *dTAK1* mutants to Gram-negative bacterial infection. In mammals, experiments with tissue cultured cells indicate a role for TAK1 in the IL1-R/TLR-NF- κ B pathway that regulates innate immune response. These data, and the results of our genetic studies of flies, suggest that the role of this MAPKKK in the control of the rel/NF- κ B innate immune response has been conserved during evolution, thereby strengthening the parallels between the regulation of mammalian and *Drosophila* innate immune responses. The list of common factors used by flies and mammals to regulate rel/NF- κ B proteins has increased in the last few years, but as shown for *dTAK1* here, it appears that the regulators that function in the IL1/TLR pathway in mammals function independently in either the Toll or Imd in *Drosophila*. The existence of two distinct pathways for regulating NF- κ B in flies may allow for the production of specific subsets of antimicrobial peptides against different pathogens (Khush et al. 2001).

We identified *dTAK1* in a screen for X-linked genes in *Drosophila* that are required to resist Gram-negative bacterial injection. Our findings illustrate the power of *Drosophila* genetics, since survival against challenge is a simple screen for genes involved in the *Drosophila* immune response. However, it is striking that among the 2500 mutagenized lines that we tested for susceptibility to Gram-negative bacterial infection, only mutations in *Dredd* and *dTAK1*, two components of the Imd pathway, were identified. This result suggests that the Imd pathway controls the most important mechanisms for combating Gram-negative bacterial infection in flies.

Materials and methods

Drosophila strains

Canton^S flies were used as a wild-type standard. *imd*, *spz*, and *l1* alleles are described elsewhere (Lemaitre et al. 1996). *Relish*^{E20}, *Dredd*^{B118}, *DmIKK β* ^{ird5} (*ird5*¹), and *DmIKK γ* ^{key1} (*kenny*¹) are either strong or null alleles of *Relish*, *Dredd*, *DmIKK β* , and *DmIKK γ* (Hedengren et al. 1999; Leulier et al. 2000; Rutschmann et al. 2000b; Lu et al. 2001). *dTAK1*¹ (*D10*¹), *dTAK1*² (*D10*²), *dTAK1*³, and *dTAK1*⁴ are four EMS-induced mutations of *dTAK1* that were generated in a *yw* chromosome. *Diptericin-lacZ* is a *P* transgene containing a fusion between 2.2 kb of upstream sequence from the *Diptericin* gene and the coding sequences from the β -galactosidase gene (Tzou et al. 2000). *Drosomycin-GFP* is a *P* transgene containing a fusion between 2.4 kb of upstream sequence from the *Drosomycin* gene and the coding sequences from the *Green Fluorescent Protein* gene (Ferandon et al. 1998). The precise fly genotypes are: *imd*: *br*, *pr*, *imd*; *spz*^{rm7}: *spz*^{rm7}/*spz*^{rm7}; *Relish*: *Relish*^{E20}, *e*; *Dredd*^{B118}: *y,w*, *Dredd*^{B118}; *dTAK1*: *y,w*, *dTAK1*; *DmIKK β* ^{ird5}: *DmIKK β* ^{ird5}, *ca*, *Diptericin-lacZ*; *DmIKK γ* ^{key1}: *y,w*, *Diptericin-lacZ*, *Drosomycin-GFP*; *cn,bw*, *sp*, *DmIKK γ* ^{key1}. For survival studies, we used a *Relish*^{E20} stock from which the *ebony* mutation was removed, because *ebony* affects survival levels (Lemaitre et al. 1996). *Drosophila* stocks were maintained at 25°C. After infection, flies

were incubated at either 25°C for Northern blots or 29°C for survival assays.

Mutagenesis

y, w male flies were treated with 25 mM EMS. The mutagenized males were crossed with females *C(1)DX y, w, f*, and single *F*₁ males were back-crossed to the same female stock to establish independent lines. Six *F*₂ males from each line were pricked with a needle dipped in a concentrated pellet of *E. carotovora* 15 (OD = 200), and incubated at 29°C overnight. Lines that showed more than four dead flies out of six were retested. Canton^S and *imd* flies were used as internal controls. For each selected line, a homozygous stock was established by crossing *F*₂ males with *FM3/1(1)44ter* females. Survival after Gram-negative bacterial infection was sufficiently reliable to establish complementation groups. Initial mapping was performed by monitoring resistance to *E. carotovora* 15 infection in recombinants between the mutated chromosome and a chromosome carrying the *y*⁺, *cv*, *f* markers. Additional mapping was done with the X chromosome deficiency kit from the Bloomington stock center.

Infection experiments

Bacterial infections were performed by pricking third instar larva or adults with a thin needle previously dipped into a concentrated culture (O.D ~200) of *E. coli*, *M. luteus*, or a mixture of the two bacteria. Natural infections with *B. bassiana* were performed by shaking anesthetized flies for thirty seconds in a Petri dish containing a sporulating fungal culture (Lemaitre et al. 1997). Bacterial and fungal strains were previously described (Lemaitre et al. 1997). Natural infection by *E. carotovora* 15 were performed by incubating *Drosophila* larvae in a mixture of crushed banana and bacteria (Basset et al. 2000).

Overexpression studies

UAS-dTAK1 and *UAS-dTAK1-K46R* have been described previously (Takatsu et al. 2000). *UAS-dTAK1-K46R* encodes a dominant negative form of *dTAK1* in which lysine 46 was replaced by an arginine. The full-length *Dredd* isoform δ^* cDNA was cloned as a *XhoI*-*Bam*HI fragment from *pMT-Dredd δ^* -Myc* (Rodriguez et al. 1999) into *XhoI*-*Bam*HI digested *pUAS* vector. *W¹¹¹⁸* flies were transformed using *P* element-mediated transformation. The *yolk-GAL4* driver strongly expresses GAL4 in the female adult fat body (J.M. Reichhart, pers. comm.). The *hsp70-GAL4* driver expresses GAL4 ubiquitously after heat shock.

Northern blot analysis

Total RNA extraction and Northern blotting experiments were performed as described (Lemaitre et al. 1997).

Sequencing of *dTAK1* alleles

Genomic DNA from the *y,w* strain and the four *dTAK1* mutants was extracted from single adult flies as described (Gloor and Engels 1992). One microliter of genomic DNA was used for each PCR amplification. Specific oligonucleotide primers for the *dTAK1* gene were synthesized and used to amplify overlapping fragments of the *dTAK1* coding sequence. The resulting single-fragment PCR products were purified using a Qiagen purification column and sequenced using the BigDye Terminator Cycle sequencing ready reaction (PE Applied Biosystems). The sequence profiles were analyzed on Edit View 1.0.1 ABI Prism

(PE Applied Biosystems) and compared to the *dTAK1* cDNA sequence published previously.

Cloning and transformation of P[dTAK1]

A P1 *Drosophila* filter (Genome Systems) was hybridized with a ³²P-labeled 3.5 kb *dTAK1* cDNA probe. Three clones were isolated, and one of them (DS08905) was used for the following experiments. The *dTAK1* coding region (10.0 kb and 5.8 kb *Bam*H1 fragments and a 2.6 kb *Eco*RI fragment) was subcloned into pBlueScriptII KS+ and sequenced using synthetic sequence primers. *Drosophila* Genome Project sequencing data, AC014558, (BDGP) was also used to confirm the genomic sequencing. A 15 kb genomic fragment that contains *dTAK1* coding region as well as 2.5 kb 5'- and 4.8 kb 3'-untranslated regions were amplified by PCR with Takara LA Taq (Takara Biomedical) by using PCR primers. This genomic fragment was digested with *Xho*I and *Not*I and then subcloned into the *Drosophila* transformation vector P[CaSpeR-4]. Flies bearing transgenes were generated by general P-element mediated transformation.

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References

Aderem, A. and Ulevitch, R.J. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* **406**: 782–787.

Anderson, K.V. 2000. Toll signaling pathways in the innate immune response. *Curr. Opin. Immunol.* **12**: 13–19.

Basset, A., Khush, R., Braun, A., Gardan, L., Boccard, F., Hoffmann, J., and Lemaitre, B. 2000. The phytopathogenic bacterium, *Erwinia carotovora*, infects *Drosophila* and activates an immune response. *Proc. Natl. Acad. Sci.* **97**: 3376–3381.

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

Belvin, M.P. and Anderson, K.V. 1996. A conserved signaling pathway: The *Drosophila* toll-dorsal pathway *Annu. Rev. Cell Dev. Biol.* **12**: 393–416.

Brand, A.H. and Perrimon, N. 1993. Targeted gene expression as a mean of altering cell fates and generating dominant phenotypes. *Development* **118**: 401–415.

Consortium, T.F. 1998. FlyBase—A *Drosophila* database. *Nucleic Acids Res.* **26**: 85–88.

Elrod-Erickson, M., Mishra, S., and Schneider, D. 2000. Interactions between the cellular and humoral immune responses in *Drosophila*. *Curr. Biol.* **10**: 781–784.

Engstrom, Y. 1999. Induction and regulation of antimicrobial peptides in *Drosophila*. *Dev. Comp. Immunol.* **23**: 345–358.

Ferrandon, D., Jung, A.C., Criqui, M., Lemaitre, B., Uttenweiler-Joseph, S., Michaut, L., Reichhart, J., and Hoffmann, J.A. 1998. A Drosomycin-GFP reporter transgene reveals a local immune response in *Drosophila* that is not dependent on the Toll pathway. *EMBO J.* **17**: 1217–1227.

Gloor, G.B. and Engels, W.R. 1992. Single-fly DNA preps for PCR. *Drosoph. Inf. Serv.* **75**: 148–149.

Han, Z.S. and Ip, Y.T. 1999. Interaction and specificity of Rel-related proteins in regulating *Drosophila* immunity gene expression. *J. Biol. Chem.* **274**: 21355–21361.

Hedengren, M., Asling, B., Dushay, M.S., Ando, I., Ekengren, S., Wihlborg, M., and Hultmark, D. 1999. Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. *Mol. Cell.* **4**: 827–837.

Hoffmann, J. and Reichhart, J. 1997. *Drosophila* immunity. *Trends Cell. Biol.* **7**: 309–316.

Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., and Ezekowitz, R.A. 1999. Phylogenetic perspectives in innate immunity. *Science* **284**: 1313–1318.

Irie, T., Muta, T., and Takeshige, K. 2000. TAK1 mediates an activation signal from toll-like receptor(s) to nuclear factor- κ B in lipopolysaccharide-stimulated macrophages. *FEBS Lett.* **467**: 160–164.

Ishitani, T., Ninomiya-Tsuji, J., Nagai, S., Nishita, M., Menechini, M., Barker, N., Waterman, M., Bowerman, B., Clevers, H., Shibuya, H., et al. 1999. The TAK1-NLK-MAPK-related pathway antagonizes signalling between β -catenin and transcription factor TCF. *Nature* **399**: 798–802.

Khush, R.S. and Lemaitre, B. 2000. Genes that fight infection: What the *Drosophila* genome says about animal immunity. *Trends Genet.* **16**: 442–449.

Khush, R.S., Leulier, F., and Lemaitre, B. 2001. *Drosophila* immunity: Two paths to NF- κ B. *Trends Immunol.* **22**: 260–264.

Kim, Y.S., Han, S.J., Ryu, J.H., Choi, K.H., Hong, Y.S., Chung, Y.H., Perrot, S., Raibaud, A., Brey, P.T., et al. 2000. Lipopolysaccharide-activated kinase, an essential component for the induction of the antimicrobial peptide genes in *Drosophila melanogaster* cells. *J. Biol. Chem.* **275**: 2071–2079.

Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., Meister, M., Georgel, P., Reichhart, J., and Hoffmann, J. 1995. A recessive mutation, immune deficiency (*imd*), defines two distinct control pathways in the *Drosophila* host defense. *Proc. Natl. Acad. Sci.* **92**: 9365–9469.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J., and Hoffmann, J. 1996. The dorsoventral regulatory gene cassette *spätzle*/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* **86**: 973–983.

Lemaitre, B., Reichhart, J., and Hoffmann, J. 1997. *Drosophila* host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc. Natl. Acad. Sci.* **94**: 14614–14619.

Leulier, F., Rodriguez, A., Khush, R.S., Chen, P., Abrams, J.M., and Lemaitre, B. 2000. The *Drosophila* caspase Dredd is required to resist Gram-negative bacterial infection. *EMBO Rep.* **1**: 353–358.

Levashina, E., Ohresser, S., Lemaitre, B., and Imler, J. 1998. Two distinct pathways can control expression of the *Drosophila* antimicrobial peptide Metchnikowin. *J. Mol. Biol.* **278**: 515–527.

Lu, Y., Wu, L.P., and Anderson, K.V. 2001. The antibacterial arm of the *Drosophila* innate immune response requires an I κ B kinase *Genes & Dev.* **15**: 104–110.

Manfrulli, P., Reichhart, J.M., Steward, R., Hoffmann, J.A., and 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.

Vidal et al.

- Meng, X., Khanuja, B.S., and Ip, Y.T. 1999. Toll receptor-mediated *Drosophila* immune response requires Dif, an NF- κ B factor. *Genes & Dev.* **13**: 792–797.
- Mihaly, J., Kockel, L., Gaengel, K., Weber, U., Bohmann, D., and Mlodzik, M. 2001. The role of the *Drosophila* TAK homologue dTAK during development. *Mech. Dev.* **102**: 67–79.
- Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, et al. 1996. A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J. Biol. Chem.* **271**: 13675–13679.
- Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. 1999. The kinase TAK1 can activate the NIK-I κ B as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* **398**: 252–256.
- Onfelt Tingvall, T., Roos, E., and Engstrom, Y. 2001. The *imd* gene is required for local Cecropin expression in *Drosophila* barrier epithelia. *EMBO Rep.* **2**: 239–243.
- Rodriguez, A., Oliver, H., Zou, H., Chen, P., Wang, X., and Abrams, J.M. 1999. Dark is a *Drosophila* homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nat. Cell Biol.* **1**: 272–279.
- Rutschmann, S., Jung, A.C., Hetru, C., Reichhart, J.M., Hoffmann, J.A., and 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, A.C., Zhou, R., Silverman, N., Hoffmann, J.A., and Ferrandon, D. 2000b. Role of *Drosophila* IKK γ in a Toll-independent antibacterial immune response. *Nat. Immunol.* **1**: 342–347.
- Sakurai, H., Miyoshi, H., Toriumi, W., and Sugita, T. 1999. Functional interactions of transforming growth factor β -activated kinase 1 with I κ B kinases to stimulate NF- κ B activation. *J. Biol. Chem.* **274**: 10641–10648.
- Sakurai, H., Shigemori, N., Hasegawa, K., and Sugita, T. 1998. TGF- β -activated kinase 1 stimulates NF- κ B activation by an NF- κ B-inducing kinase-independent mechanism. *Biochem. Biophys. Res. Commun.* **243**: 545–549.
- Shibuya, H., Iwata, H., Masuyama, N., Gotoh, Y., Yamaguchi, K., Irie, K., Matsumoto, K., Nishida, E., and Ueno, N. 1998. Role of TAK1 and TAB1 in BMP signaling in early *Xenopus* development. *EMBO J.* **17**: 1019–1028.
- Shirakabe, K., Yamaguchi, K., Shibuya, H., Irie, K., Matsuda, S., Moriguchi, T., Gotoh, Y., Matsumoto, K., and Nishida, E. 1997. TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. *J. Biol. Chem.* **272**: 8141–8144.
- Silverman, N., Zhou, J., Stöven, S., Pandey, N., Hultmark, D., and Maniatis, T. 2000. A *Drosophila* I κ B kinase complex required for Relish cleavage and antibacterial immunity. *Genes & Dev.* **14**: 2461–2471.
- Stöven, S., Ando, I., Kadalayil, L., Engström, Y., and Hultmark, D. 2000. Activation of the *Drosophila* NF- κ B factor Relish by rapid endoproteolytic cleavage. *EMBO Rep.* **1**: 347–352.
- Takatsu, Y., Nakamura, M., Stapleton, M., Danos, M.C., Matsumoto, K., O'Connor, M.B., Shibuya, H., and Ueno, N. 2000. TAK1 participates in c-Jun N-terminal kinase signaling during *Drosophila* development. *Mol. Cell. Biol.* **20**: 3015–3026.
- Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J.M., Lemaitre, B., Hoffmann, J.A., and Imler, J.L. 2000. Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. *Immunity* **13**: 737–748.
- Wang, W., Zhou, G., Hu, M.C., Yao, Z., and Tan, T.H. 1997. Activation of the hematopoietic progenitor kinase-1 (HPK1)-dependent, stress-activated c-Jun N-terminal kinase (JNK) pathway by transforming growth factor- β (TGF- β)-activated kinase (TAK1), a kinase mediator of TGF- β signal transduction. *J. Biol. Chem.* **272**: 22771–22775.
- Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. 1995. Identification of a member of the MAPKKK family as a potential mediator of TGF- β signal transduction. *Science* **270**: 2008–2011.