The MAPKKK Mekk1 regulates the expression of *Turandot* stress genes in response to septic injury in *Drosophila*

Sylvain Brun[1,†], Sheila Vidal[1,†], Paul Spellman[2], Kuniaki Takahashi[3], Hervé Tricoire[4] and Bruno Lemaitre[1,*]

[1]*Centre de Génétique Moléculaire, CNRS, 91198 Gif-sur-Yvette, France*
[2]*Department of Molecular and Cell Biology and Howard Hughes Medical Institute, University of California at Berkeley, Berkeley, CA 94720-3200, USA*
[3]*National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan*
[4]*Institut Jacques Monod, 2 place Jussieu, 75251 Paris, France*

Septic injury triggers a rapid and widespread response in *Drosophila* adults that involves the up-regulation of many genes required to combat infection and for wound healing. Genome-wide expression profiling has already demonstrated that this response is controlled by signaling through the Toll, Imd, JAK-STAT and JNK pathways. Using oligonucleotide microarrays, we now demonstrate that the MAPKKK Mekk1 regulates a small subset of genes induced by septic injury including *Turandot* (*Tot*) stress genes. Our analysis indicates that Tot genes show a complex regulation pattern including signals from both the JAK-STAT and Imd pathways and Mekk1. Interestingly, *Mekk1* flies are resistant to microbial infection but susceptible to paraquat, an inducer of oxidative stress. These results point to a role of Mekk1 in the protection against tissue damage and/or protein degradation and indicate complex interactions between stress and immune pathways in *Drosophila*.

Introduction

Innate immune responses are regulated by evolutionary conserved signaling cascades. Analyzing the contribution of each cascade to the control of immune-responsive genes is a major challenge to dissect animal host defense mechanisms. In *Drosophila*, four distinct pathways, Toll, Imd, JNK and JAK-STAT have been implicated in the regulation of genes induced after bacterial challenge (De Gregorio *et al*. 2001; Irving *et al*. 2001; Boutros *et al*. 2002; Silverman *et al*. 2003).

Genetic analyses have shown that anti-microbial peptide encoding genes are regulated by the Toll and Imd pathways (Tzou *et al*. 2002; Hoffmann 2003; Hultmark 2003). These two pathways share similarities with the Toll-Like Receptor and Tumor Necrosis Factor Receptor pathways, respectively, which regulate NF-κB in mammals. The Toll pathway is activated mainly by Gram-positive bacteria and fungi, while the Imd pathway responds mainly to Gram-negative bacterial infection. Microarray analyses have shown that the Toll and Imd cascades control the majority of genes regulated by septic injury in addition to anti-microbial peptide encoding genes. The presence of immune-responsive genes independent or only partially dependent on both the Imd and Toll pathways suggested the involvement of other signaling cascades (De Gregorio *et al*. 2002).

Using a similar approach, Boutros *et al*. (2002) have shown that in addition to the Toll and Imd pathways, the JAK-STAT and the JNK pathways contribute to the expression of immune response genes. The *Drosophila* JAK-STAT pathway is involved in multiple developmental events and regulates hemocyte differentiation (Dearolf 1999; Agaisse & Perrimon 2004). This pathway does not regulate anti-microbial peptide genes but affects the expression of a small number of genes induced in the fat body after septic injury (Lagueux *et al*. 2000). Recently, it has been shown that the JAK-STAT pathway is activated in the fat body by the cytokine Unpaired3 (Upd3), and that it regulates the expression of *Turandot* (*Tot*) stress genes in response to septic injury (Agaisse *et al*. 2003).

In *Drosophila*, the JNK Mitogen Activated Protein Kinase (MAPK) pathway is induced following immune stimulation (Sluss *et al*. 1996). Interestingly, both NF-κB and JNK

†These authors contributed equally to this work.
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**Correspondence*: E-mail: lemaitre@cgm.cnrs-gif.fr

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branches share the same upstream components, Tak1 and Imd, indicating that the activation of both cascades is tightly linked in *Drosophila* (Boutros *et al.* 2002; Silverman *et al.* 2003; Park *et al.* 2004). Furthermore, in agreement with a function in wound healing (Rämet *et al.* 2001; Boutros *et al.* 2002; Galko & Krasnow 2004), genome profiling indicates that JNK signaling controls the expression of genes involved in cytoskeleton remodeling.

In plants, *C. elegans* and vertebrates, p38 MAPKs are also involved in the regulation of the immune and stress responses (Asai *et al.* 2002; Kim *et al.* 2002). However, little is known about the role of this pathway in the *Drosophila* immune response. Two p38 MAP Kinases, p38a and p38b, are encoded by the *Drosophila* genome (Han *et al.* 1998b). Like mammalian p38, *Drosophila* p38s are activated in cell culture by stress and inflammatory stimuli, such as UV radiation, high osmolarity, heat-shock, serum starvation and bacterial products (Han *et al.* 1998a; Zhuang *et al.* 2005). Flies lacking *p38a* are viable but are susceptible to some environmental stresses, including heat-shock, oxidative stress and starvation (Craig *et al.* 2004). However, the precise physiological role of p38 awaits further studies using loss-of-function mutations of the second p38 gene, *p38b*. *Drosophila* Mekk1 is a MAPK Kinase Kinase (MAPKKK) similar to the mammalian MEKK4/MTK1. *Drosophila* mutants lacking *Mekk1* show a reduced activation of p38 *in vivo* and are hypersensitive to some environmental stresses such as elevated temperature and increased osmolarity, suggesting that the Mekk1-p38 pathway is critical for the response to environmental stress in *Drosophila* (Inoue *et al.* 2001).

In the present study, we have analyzed the role of Mekk1 in the regulation of genes induced in response to septic injury. Our study demonstrates that the MAPKKK Mekk1 regulates a small subset of target genes induced by septic injury, including *Turandot* stress genes. Furthermore, *Mekk1* mutant flies are susceptible to oxidative stress, suggesting a role of this MAPKKK in the protection against tissue damage and/or protein degradation.

Results

Identification of Mekk1 target genes using oligonucleotide microarrays

In a previous study, we have identified 400 *Drosophila* immune regulated genes (DIRGs) through a microarray analysis of the transcriptome after septic injury and natural infection (De Gregorio *et al.* 2001). To identify whether some of the 400 previously identified DIRGs are controlled by Mekk1, total RNA samples from wild-type (Oregon R) and *Mekk1* adult flies, collected after septic injury with a mixture of *E. coli* and *Micrococcus luteus* were hybridized to Affymetrix DrosGenome1 GeneChips capable of measuring RNA levels for nearly every gene in the *Drosophila* genome. Changes in relative transcript levels were measured 1.5, 3, 6, 12, 24 and 48 h after septic injury, ensuring that both early and late gene changes were monitored. Each time series was performed in duplicate for challenged and quadruplet for unchallenged flies. Complete results can be found at: http://www.cgm.cnrs-gif.fr/immunity/enindex.html

Table 1 shows a list of genes that display a change in their expression between wild-type and *Mekk1* flies using a two-fold threshold. We found that Mekk1 does not regulate anti-microbial peptides encoding genes, but affects a small group of genes that could not be simply clustered to only one biological function. This list includes the gene coding for the Thiol-Ester Protein II, *Tep*II, which may participate in microbial opsonization (Levashina *et al.* 2001). Interestingly, the most significantly affected gene was *Turandot M* (*TotM*) which was induced seven-fold in wild-type but not in *Mekk1* flies (Fig. 1A). *TotM*

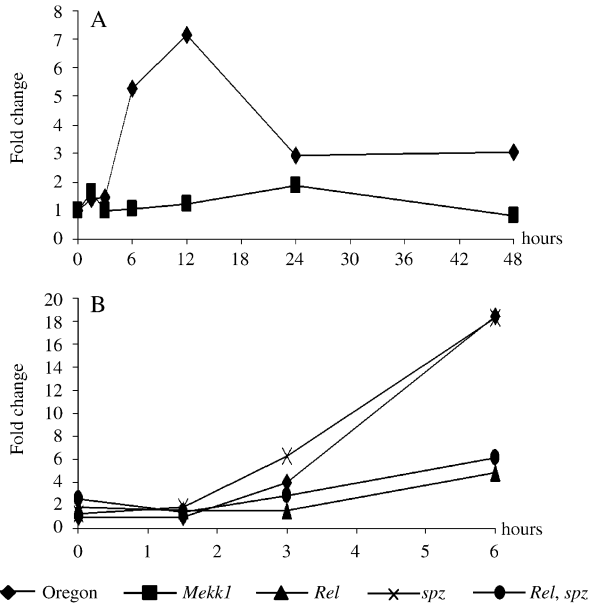

Figure 1 Determination of the *TotM* expression profile by oligonucleotide microarrays. (A) The expression profile of *TotM* in wild-type (Oregon R) and *Mekk1* adult males in response to septic injury with a mixture of *E. coli* and *M. luteus* was monitored by microarray analysis. The graph indicates fold changes of RNA expression levels compared with uninfected flies at specific times after infection (0–48 h). (B) The graph shows the expression profile of *TotM* in response to septic injury (time 0–6 h) in wild-type (Oregon R), *Rel*, *spz* and *Rel*, *spz* double mutant flies. Data were obtained from De Gregorio *et al.* (2002).

Table 1 Immune response genes affected in Mekk1 mutants

	Oregon R time after septic injury							*Mekk1* time after septic injury							
Name	C	1.5 h	3 h	6 h	12 h	24 h	48 h	C	1.5 h	3 h	6 h	12 h	24 h	48 h	Domain/function
Repressed:															
CG30080	1.0	14.6	14.5	15.6	8.9	10.4	6.5	0.8	**1.6**	**3.3**	**3.2**	**2.2**	**2.0**	**2.4**	small peptide
TotM	1.0	1.4	1.5	5.2	7.1	2.9	3.1	1.0	1.6	1.0	**1.0**	**1.2**	1.9	0.8	stress response
CG9989	1.0	2.4	1.6	1.5	1.9	2.6	2.8	1.4	**1.2**	1.1	1.2	**1.1**	**1.2**	1.0	endonuclease
TepII	1.0	2.6	4.6	13.0	9.7	6.3	4.4	0.8	3.3	2.8	**3.3**	**3.2**	**1.2**	**1.7**	humoral defense
CG14957	1.0	4.6	6.5	2.6	1.5	0.9	1.2	1.3	2.9	**1.4**	**1.3**	1.5	1.2	1.1	chitin binding domain
CG3604	1.0	1.3	3.6	2.7	1.6	1.0	1.5	0.8	1.0	**1.5**	**1.1**	**0.8**	1.6	1.2	protease inhibitor
CG13905	1.0	6.0	8.9	7.1	3.6	2.9	1.9	1.4	**2.3**	**2.9**	**2.9**	2.6	1.2	1.2	unknown
CG15043	1.0	3.6	3.6	2.4	1.8	1.9	2.6	0.7	2.1	**1.3**	1.3	1.1	**0.9**	**1.0**	unknown
CG10912	1.0	2.5	2.2	1.7	1.3	1.1	1.6	0.7	**1.2**	**1.0**	0.9	1.0	0.7	**0.7**	unknown
CG4950	1.0	1.0	3.2	1.1	1.2	0.8	1.3	1.3	1.2	**1.2**	0.6	0.7	1.3	2.0	leucin rich
CG15829	1.0	2.9	2.7	1.8	1.0	1.3	1.0	1.4	**1.4**	**1.1**	1.2	1.3	1.3	1.0	acyl-coA binding
minA	1.0	7.6	2.1	1.0	0.9	1.1	1.0	0.7	**2.9**	1.5	0.7	0.8	0.7	0.6	adhesion molecule
CG15292	1.0	0.5	1.0	2.6	1.6	0.9	0.4	1.2	1.0	1.5	**0.7**	**0.7**	1.5	0.4	unknown
CG7296	1.0	2.3	3.8	2.3	1.1	1.3	1.4	1.5	1.7	**1.7**	1.8	1.4	1.3	1.3	unknown
DNaseII	1.0	0.8	0.9	1.2	3.8	5.7	8.3	1.3	1.0	0.7	1.2	**1.0**	**1.6**	**1.4**	deoxyribonuclease
Induced:															
Def	1.0	1.2	7.6	18.0	10.9	5.5	2.4	0.8	**3.8**	**20.5**	**41.4**	**34.2**	**13.7**	**6.4**	antibacterial peptide
CG5778	1.0	1.1	1.6	2.0	3.9	4.2	5.1	2.1	3.2	**3.2**	**4.6**	5.7	**9.0**	7.9	unknown
Tsf1	1.0	1.0	1.0	1.7	2.1	6.1	9.5	1.3	**2.0**	**2.4**	3.2	3.3	9.3	11.3	iron binding
CG15281	1.0	0.8	0.3	0.4	0.9	0.9	1.9	3.6	**2.9**	**1.8**	**1.2**	1.9	1.7	3.4	unknown
Uro	1.0	3.1	2.7	3.1	2.2	1.8	2.1	3.9	**7.2**	**6.4**	5.2	**5.5**	**4.0**	**4.3**	urate oxidase

The numbers indicate relative expression levels compared to unchallenged control flies (C). Changes greater than two-fold are indicated in bold. Top: genes repressed in *Mekk1* flies; Bottom: genes up-regulated in *Mekk1* flies.

belongs to a family of eight *Tot* genes that encode small secreted proteins of 11–14 kDa sharing weak homology (Ekengren & Hultmark 2001). Previous studies have shown that these genes are induced under stress conditions such as bacterial infection, heat-shock, paraquat feeding and UV exposure, suggesting a role in stress tolerance in *Drosophila* (Ekengren *et al*. 2001). Table 1 also shows that Mekk1 represses the expression of a low number of immune genes including *Defensin*.

Mekk1 regulates *Tot* genes in response to septic injury

The best characterized member of the Turandot family is TotA which is secreted by the fat body and accumulates in the hemolymph in response to various stress stimuli (Ekengren *et al*. 2001). *TotA* was not predicted in the first release of the *Drosophila* genome and was in consequence not included on the DrosGenome1 GeneChips. To confirm the microarray results, we monitored the mRNA levels of *TotA* and *TotM* in *Mekk1* flies by Northern blot analysis. Pricking wild-type flies with a mixture of Gram-positive and Gram-negative bacteria strongly activated the expression of *TotM* and *TotA*, whereas in *Mekk1* mutants, *Tot* genes expression was dramatically reduced (Fig. 2A,B). However, *Diptericin* and *Drosomycin* which encode anti-microbial peptides were induced at a wild-type level in *Mekk1* flies. These RNA blots strengthen the microarray analysis and show that induction of both *TotA* and *TotM* after septic injury requires Mekk1. To confirm that the lack of induction of *TotA* was indeed due to the *Mekk1* mutation, we performed a rescue experiment with a *hsp-Mekk1* construct. Figure 2B clearly shows that over-expression of the *Mekk1* cDNA after heat-shock restored a wild-type level of *TotA* expression in *Mekk1* mutants after pricking. In agreement with a previous study (Agaisse *et al*. 2003), we observed that the basal levels in unchallenged flies and the induction levels after septic injury of *TotA* expression significantly vary depending on age of the flies, growth conditions and also the genetic background. Therefore, we also exploited the inducible RNA interference technology (RNAi) as an alternative way to analyze the phenotype associated with the knock-down of *Mekk1*. We generated two

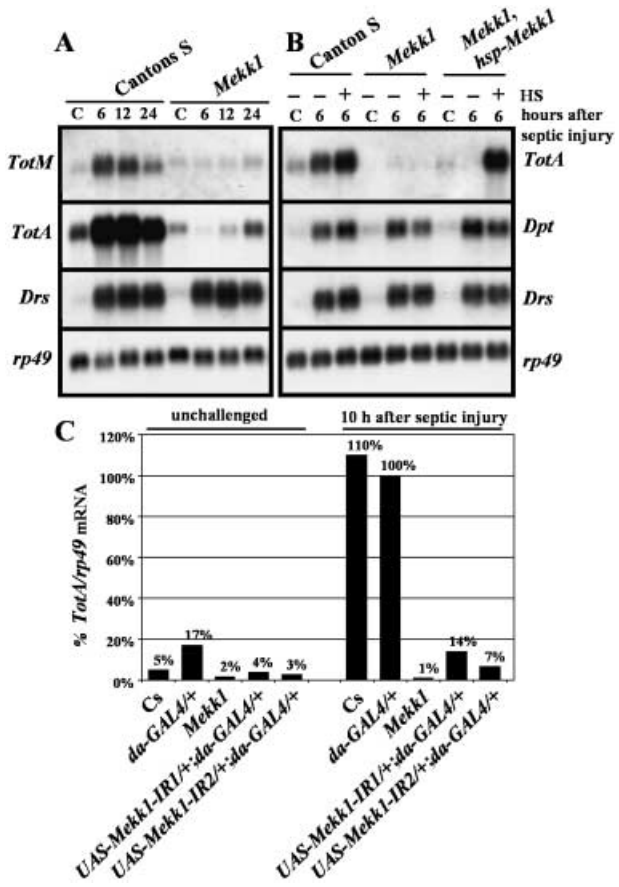

Figure 2 Expression of *TotA* and *TotM* genes in *Mekk1* adults after septic injury. (A) A time course of *TotA*, *TotM* and *Drs* gene expression in wild-type (Canton S) and *Mekk1* mutants infected with a mixture of *E. coli* and *M. luteus*, shows that *Mekk1* controls the expression of *TotA* and *TotM*. (B) Northern blot analysis of total RNA extracts from adult flies collected 6 h after septic injury with a mixture of *M. luteus* and *E. coli*. The data show that a high level of *TotA* expression is observed in Canton S flies as well as in *Mekk1*, *hsp-Mekk1* flies collected after heat-shock. A one hour heat-shock (37 °C) was performed just before infecting the flies. Over-expression of the *Mekk1* cDNA after heat-shock induce a moderate level of *TotA* expression in wild-type in absence of challenge (data not shown). (C) Unchallenged flies. (HS) Heat-shock treatment. *rp49* mRNA was used as internal control. *Dpt*: *Diptericin*; Drs: *Drosomycin*. (C) Quantitative RT-PCR analysis of RNA samples extracted from adult flies shows that the induction of *TotA* is reduced in flies that contained both the *UAS-DMekk1-IR* element and the *da-GAL4* driver. Canton S and *da-GAL4/+* flies were used as wild-type controls. 100% represents level of expression of *TotA* after septic injury in *da-GAL4/+* flies. Flies were collected at 10 h after septic injury. Two independent *UAS-DMekk1-IR* (1 and 2) insertion lines were used.

independent transgenic fly lines, *UAS-Mekk1-IR*, containing a *GAL4*-inducible construct which allows the tissue-specific production of double-stranded RNA (dsRNA) of *Mekk1*. Figure 2C clearly shows that silencing of *Mekk1*, using the ubiquitous driver *daughterless-GAL4* (*da-GAL4*) blocked *TotA* expression after septic injury. In an attempt to determine in which tissue Mekk1 is required for *TotA* expression, we directed *Mekk1* dsRNA synthesis in the fat body using the *ppl-GAL4* and in the hemocytes using the *hml-GAL4* driver. While we did not observe any effect using *hml-GAL4*, knock-down of *Mekk1* in the fat body using the *ppl*-GAL4 driver reduced *TotA* expression, suggesting that Mekk1 is required in the fat body but not in hemocytes (data not shown).

Mekk1 regulates *Tot* genes in response to stresses

As stated above, *Tot* genes are induced by various stresses and after septic injury. To determine if Mekk1 also regulates *TotA* under stress conditions, we compared the level of *TotA* transcripts after heat-shock, dehydration, mechanical pressure, and osmotic stress in wild-type and in *Mekk1* flies. In these experiments, heat-shock, dehydration and mechanical pressure weakly induced *TotA* expression, whereas osmotic stress had no effect (Fig. 3A). Nevertheless, the weak stimulation of *TotA* by stress was clearly abolished in *Mekk1* flies. Septic injury was by far the strongest and most reliable inducer of *TotA* (Fig. 3A). Notably, Fig. 3C shows that Gram-negative bacteria induced *TotA* expression at a higher level than Gram-positive bacteria in agreement with a previous study (Agaisse *et al*. 2003). However, we observed that an injury without addition of bacteria induced *TotA* at 40% of the level of septic injury by Gram-negative bacteria. This expression profile significantly differed from that of the anti-bacterial peptide gene *Diptericin*, which is only weakly induced by clean injury (Lemaitre *et al*. 1997 and Fig. 3C). This suggests that *TotA* is induced by a stimulus associated with the injury itself, which is enhanced in presence of Gram-negative bacteria. Consistent with this idea, we observed that *TotA* was only weakly induced after natural infection of adults by the entomopathogenic fungus *B. bassiana* (Fig. 3B). Similarly, *TotA* was not induced after natural infection with the Gram-negative bacteria *Erwinia carotovora 15* in larvae (data not shown). Taken together, these findings suggest that Mekk1 is mostly activated by the stress of the injury (wound, oxidative or mechanical stress), and that the higher induction of *Tot* genes after septic injury is conferred by the concurrent activation of other pathways triggered by the presence of microbial components.

Relationship between Mekk1 and the JAK-STAT pathway

Recently it has been shown that septic injury activates *TotA* expression through the Imd and the JAK-STAT

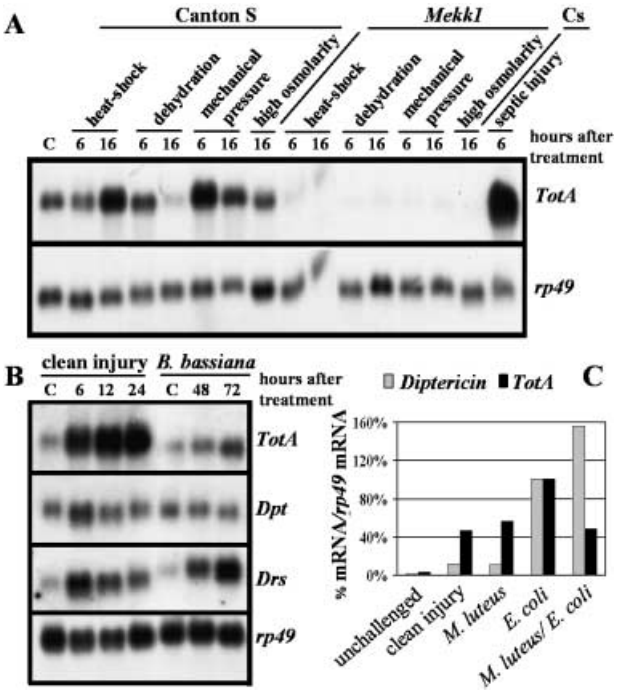

Figure 3 Expression of *TotA* in *Mekk1* adults in response to different stress conditions. (A) Northern blot analysis of total RNA extracted from wild-type and *Mekk1* adult flies collected after various stresses (heat-shock, dehydration, mechanical pressure, high osmolarity). Flies were collected 6 or 16 h after the start of the treatment. Septic injury was performed with a mixture of *E. coli* and *M. luteus*. (B) Northern blot analysis of total RNA extracts from Canton S adult flies shows that *TotA* expression is highly induced after simple injury, whereas it is weakly induced after natural infection by *B. bassiana*. *rp49* mRNA was used as internal control. *Dpt*: *Diptericin*; Drs: *Drosomycin*. (C) Quantitative RT-PCR analysis of RNA extracted from Canton S flies 11 h after injury shows that clean injury alone induces more *TotA* expression than *Diptericin* expression compared to expression of both genes after septic injury. For *Department* and *TotA* expression, 100% represents the level of expression after *E. coli* infection.

pathways (Agaisse *et al.* 2003). We confirmed that *Tot* gene induction is clearly blocked in *Relish* deficient flies that lack a functional Imd pathway but not in *Toll* deficient flies (Fig. 1B for *TotM* and Fig. 4A for *TotA*). However, Mekk1 is unlikely to participate in the Imd pathway, since in contrast to mutations of the MAPKKK *Tak1*, *Mekk1* loss-of-function does not affect *Diptericin* expression or survival after challenge with Gram-negative bacteria (Figs 2B and 5A).

To analyze the relationship between Mekk1 and the JAK-STAT pathway, we used fly lines carrying either a loss-of-function (hop^{msv1}) or a gain-of-function mutation (hop^{Tum}) in the *Drosophila* JAK kinase Hopscotch (Hop). As expected, *TotA* expression was not induced in *JAK/*hop^{msv1} deficient flies after septic injury and was expressed at a very high level in the gain-of-function mutant *JAK/*hop^{Tum} in absence of a challenge (Fig. 4B). Figure 4C shows that this high and constitutive expression of *TotA* in hop^{Tum} mutant flies is only weakly affected in flies carrying both the hop^{Tum} and *Mekk1* mutations. This last result strongly suggests that Mekk1 is not required downstream of JAK/Hop for the regulation of *Tot* genes.

The gene encoding the Upd3 cytokine is rapidly induced in hemocytes after septic injury and it is believed that secretion of Upd3 activates the JAK-STAT pathway in the fat body (Agaisse *et al.* 2003). Figure 4D shows that *upd3* expression was not affected in *Mekk1* flies after septic injury, indicating that Mekk1 is not involved in the regulation of this cytokine. This result is consistent with the observation that *Mekk1* is not required in hemocytes for *TotA* expression. Altogether, our results indicate that Mekk1 is not a canonical component of the JAK-STAT and Imd pathways. They also underline the complexity of *TotA* gene regulation that integrate signals from the JAK-STAT and Imd pathways and Mekk1.

Mekk1 protects adult flies from oxidative stress

Mekk1 regulates a small set of genes, such as *tepII*, *TotM* and *Def*, that are supposed to have important functions during immune and stress responses. We further assayed the susceptibility of flies carrying a null allele of *Mekk1* to infection by four microorganisms (Fig. 5). We pricked flies with the Gram-negative bacterium *Escherichia coli*, the Gram-positive bacterium *Enterococcus faecalis* or the fungus *Aspergillus fumigatus* and naturally infected flies with the entomopathogenic fungus *Beauveria bassiana*. As expected, a mutation in *Relish* induced a high susceptibility to *E. coli* (Fig. 5A) while a mutation in *spaetzle*, affecting the Toll pathway, rendered flies susceptible to both Gram-positive and fungal infections (Fig. 5B,C) (Lemaitre *et al.* 1996; Rutschmann *et al.* 2002). In sharp contrast, *Mekk1* flies showed a survival rate similar to wild-type in all conditions tested (Fig. 5 and data not shown for *B. bassiana* infection). This result was consistent with our observation that *Mekk1* does not affect the expression of *Drosophila* anti-microbial peptide encoding genes after microbial infection.

Many studies indicate that MAPK pathways can be activated in response to reactive oxygen species (ROS) and recent studies have indicated a role of ROS during *Drosophila* immune responses (Nappi & Vass 2001; Ha *et al.* 2005). We next investigated whether loss of *Mekk1* leads to increased sensitivity to oxidative stress provoked by paraquat feeding. In addition to *Mekk1* mutants, we

also used *UAS-Mekk1-IR* flies. Strikingly, when *Mekk1* dsRNA was produced ubiquitously with *da-GAL4*, we observed a strong increase in sensitivity to oxidative stress induced by paraquat (Fig. 6A). Similar results were obtained with *Mekk1* mutants, showing that the increased sensitivity to paraquat was not due to an effect of the genetic background. Furthermore, over-expression of *Mekk1* with a *hsp-Mekk1* construct rescued to a large extent the oversensitivity of *Mekk1* flies to paraquat compared to control flies after heat-shock (Fig. 6B). Altogether, these results clearly demonstrate that Mekk1 is required in adult flies for protection against oxidative stress induced by paraquat.

Discussion

MAPK pathways have been implicated in a variety of immune-related pathways in vertebrates, plants and *C. elegans*, but little is known on their role during the *Drosophila* immune response. In this study, we show that *Mekk1* is not essential to combat infection and does not participate in the Toll and Imd pathways. Consistent with the immune phenotype of *Mekk1* mutants, our microarray analysis indicates that the majority of the immune-responsive genes are not affected by *Mekk1*. In contrast, we observed that Mekk1 regulates a small group of genes encoding proteins with various functions. Prominent among them are the *Tot* stress genes that are induced by septic injury. Using a null mutation in *Mekk1* as well as RNAi, we confirme that Mekk1 tightly regulates the expression of *TotA* and *TotM*, two members of the *Turandot* family. Interestingly, genes regulated by Mekk1 are similar to those regulated by the JAK-STAT pathway. Our epistasis analysis indicates that Mekk1 is unlikely to be required downstream of JAK/Hop and is not required for *upd3*

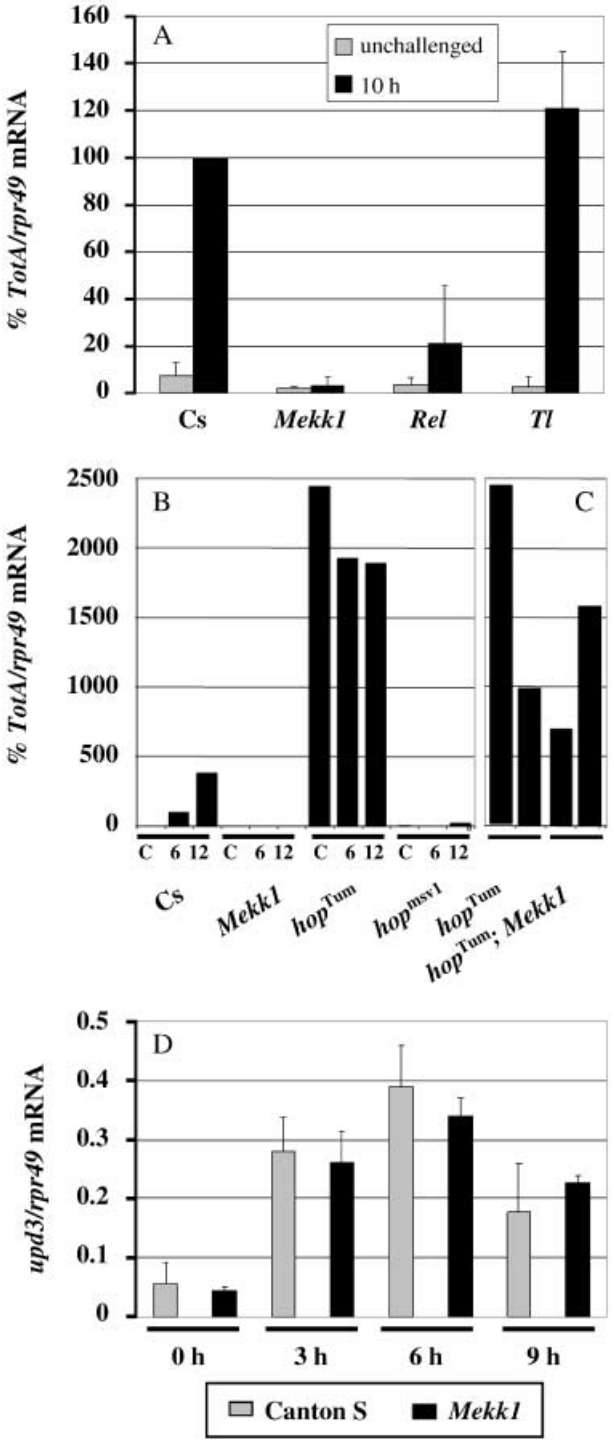

Figure 4 Relationship between Mekk1 and the JAK-STAT pathway. (A) Quantitative RT-PCR analysis of RNA samples extracted from adult flies shows that *TotA* expression after septic injury is blocked in *Mekk1* and *Rel* mutant flies, while it is not affected in *Tl* mutant flies. 100% represents *TotA* expression 10 h after septic injury in Canton S flies. (B) Quantification of *TotA* expression in wild-type (Canton S), *Mekk1*, hop^{msv1}, hop^{Tum} simple mutant and hop^{Tum}; *Mekk1* double mutant flies is expressed as the percentage of expression observed in wild-type adults at 6 h after infection. For each genotype, flies have been collected in absence of challenge (C) or 6 h and 12 h after septic injury. This Northern blot quantification shows that expression of *TotA* is induced at a high level in hop^{Tum} mutants (both after challenge and in absence of challenge), whereas *TotA* expression is abolished in *Mekk1* and hop^{msv1} mutants. (C) Two independent Northern blot quantifications show that *Mekk1* does not block the high *TotA* expression induced by hop^{Tum} in hop^{Tum}; *Mekk1* flies (unchallenged flies). (D) Quantitative RT-PCR analysis of RNA samples extracted from adult flies shows that the induction of *upd3* is not affected in *Mekk1* flies. Flies were collected 3 h, 6 h and 9 h after septic injury. Units correspond to relative copy numbers of *upd3* mRNA compared to *rp49* mRNA.

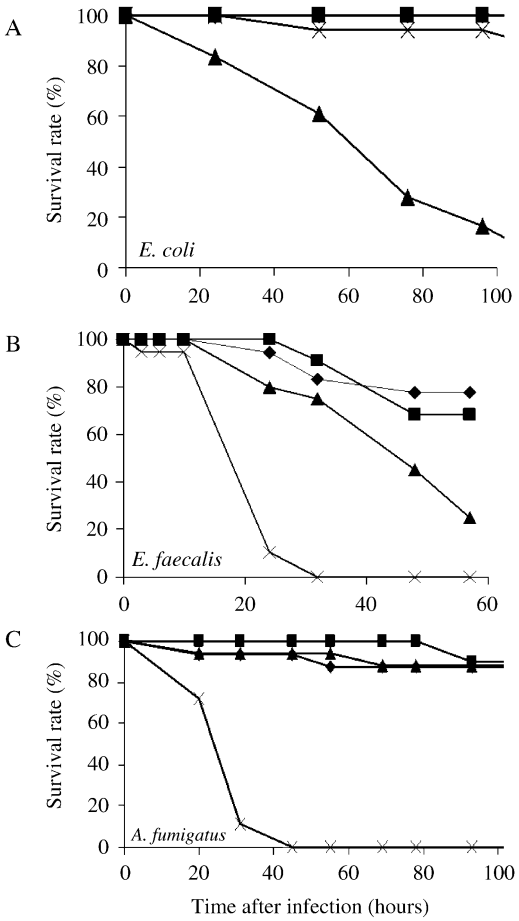

Figure 5 Resistance to microbial infection of Mekk1 flies. The survival rates of wild-type (Canton S) (◆), *Mekk1* (■), *Rel* (▲) and *spz* (×) flies after different types of infection are presented. One hundred 2–4 days old adults were pricked with a needle dipped into a suspension of one of the following microbes: (A) *E. coli*, (B) *E. faecalis* (C) *A. fumigatus*. The infected flies were incubated at 25 °C and transferred to fresh tubes 72 h after treatment.

induction in hemocytes. Although it cannot be excluded that Mekk1 functions between Upd3 and JAK/Hop, our data suggest that Mekk1 is not a canonical component of the JAK-STAT pathway. Accordingly, *Mekk1* flies are perfectly viable compared to JAK-STAT deficient mutants that are impaired in many developmental processes and show poor viability.

The exact role of Mekk1 in stress signaling remains to be investigated. At this point, we cannot exclude that Mekk1 is part of secondary cascade that branches downstream of the Imd pathway and regulates only a subset of target genes in a situation analogous to the JNK pathway which functions downstream of Tak1 (Boutros *et al.* 2002; Silverman *et al.* 2003; Park *et al.* 2004). Altogether, our work shows that *TotA* integrates input from multiple signaling pathways. It will be a major challenge to determine how these pathways interact. From this point of view, *Tot* genes define a new class of immune-inducible genes and provide an easy read-out to monitor JAK-STAT and Mekk1 activities and thus may be used to identify additional signaling components. An important question is whether Mekk1 controls *TotA* through the activation of p38 MAPKs. It has been shown *in vivo* and in cell culture that Mekk1 inhibition reduces p38 activation in *Drosophila* (Inoue *et al.* 2001; Zhuang *et al.* 2005). However, *p38a* and *Mekk1* mutants show only partially overlapping phenotypes. For instance, *p38a* but not *Mekk1* flies were vulnerable to hydrogen peroxide (Craig *et al.* 2004). It is likely that *p38b* and the existence of other MAPKKK acting upstream of p38 may account for the differences between *p38a* and *Mekk1* mutants. A more thorough depletion of p38 function *in vivo* by removing both *p38a* and *p38b* will allow clarification of the role of this family of MAPK in stress signaling.

Septic injury was the most efficient challenge to stimulate *TotA* expression. It is therefore tempting to speculate that Mekk1 and its target genes from the *Tot* family play a role in the response to tissue damage. Mekk1 has already been implicated in survival to high temperature and osmotic stress (Inoue *et al.* 2001). Here, we show a role for Mekk1 in the resistance to paraquat, an inducer of oxidative stress, in *Drosophila*. This contrasts with a previous study that indicated that *Mekk1* flies show a wild-type resistance to hydrogen peroxide (Craig *et al.* 2004). This discrepancy could be explained by the fact that in contrast to hydrogen peroxide that can exert its oxidative properties everywhere in the organism, paraquat has to be metabolized inside cells to inhibit mitochondrial complex 1 and subsequently to induce a release of superoxide ions susceptible to damage tissue. Actually, oxidative stress specificities have already been observed *in vivo*, both at genetic and molecular levels (Monnier *et al.* 2002; Girardot *et al.* 2004). Consequently, it is possible that both Mekk1 and JAK-STAT pathways mediate a host response to protect against tissue damage and protein degradation in stressful conditions. The fact that tissue damage can be caused by multiple stimuli, such as infection, injury and environmental stresses may explain the complex regulation of *Tot* genes by multiple pathways. In agreement with this hypothesis, we found that *TotA* is only weakly induced by *B. bassiana* and *E. carotovora* natural infections, which are known to trigger the Toll and Imd pathways without provoking a major injury (Lemaitre *et al.* 1997; Basset *et al.* 2000). However, the situation is probably more complex since we did not

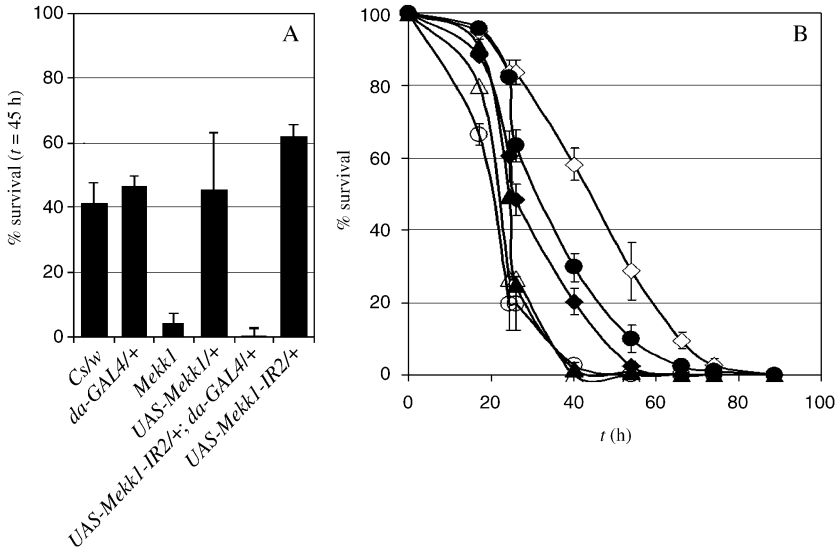

Figure 6 Susceptibility of *Mekk1* adult flies to paraquat-induced oxidative stress. (A) *Mekk1* homozygote flies and *UAS-Mekk1-IR/da-GAL4* flies showed a lower level of survival compared to control genotypes when transferred on medium containing 10 mM paraquat. Male adults were used in this experiment. (B) The survival on 10 mM paraquat medium was strongly decreased for *Mekk1* homozygote flies (△) or *Mekk1*, *hsp-Mekk1/Mekk1* flies (○) compared to *w* control flies (◇). When 37 °C heat-shocks were performed every 24 h, we observed a survival rescue for *Mekk1*, *hsp-Mekk1/Mekk1* flies (●), compared to *w* control flies (◆). By contrast the survival of *Mekk1* flies (▲) was still strongly affected. It should be noted that heat-shock decreased survival of *w* flies but increased survival of *Mekk1*, *hsp-Mekk1/Mekk1* flies. This strongly suggests that the rescue is linked to an increased protection from paraquat-induced oxidative stress by Mekk1.

observe any induction of *TotA* in adults upon paraquat feeding (data not shown). This suggests that the susceptibility of *Mekk1* mutant flies to paraquat is not directly linked to a lack of *TotA* expression. Hence, the exact role of *Tot* genes remains unknown after our study but can be further investigated through the analysis of *Mekk1* phenotypes. A significant advantage of the *Mekk1* mutation is that flies are perfectly viable under normal conditions, unlike mutations affecting the JAK-STAT pathway.

In conclusion, our study points towards a role for Mekk1 and possibly p38 in the adaptive response to stress associated with injury. The identification of target genes from the *Tot* family opens the way to genetic screens that should allow the identification of new components of the signaling cascade. *Drosophila* may provide an excellent model to study the complex interactions between stress and immune pathways.

Experimental procedures

Drosophila stocks

Oregon R, Canton S, *w* Canton S and w^{1118} flies were used as wild-type controls. Exact genotypes of the flies analyzed in this study are: $spaetzle^{rm7}$ (*spz*); $Relish^{E20}$ (*Rel*) *w*; $Mekk1^{Ur36}$ (*Mekk1*); spz^{rm7}, Rel^{E20} (*Rel*, *spz*) $Toll^{1RXA}/Toll^{R632}$ (*Tl*); hop^{msv1}, hop^{Tum} (Inoue *et al.* 2001; Lemaitre *et al.* 1996). spz^{rm7}, Rel^{E20} and $Mekk1^{Ur36}$ are strong or null alleles of *spz*, *Rel* and *Mekk1* (Hedengren *et al.* 1999). The *hop* alleles used, *msv1* and *Tum*, have been previously described (Agaisse *et al.* 2003). Rescue experiments were performed with flies carrying both the *Mekk1* mutation and an *hsp-Mekk1* P transgene on the same chromosome (Inoue *et al.* 2001). RNAi transgenic fly lines of *Mekk1* were obtained using the inducible RNAi method. A 500 bp-long cDNA fragment (nucleotides 1–500 of the coding sequence) was amplified by PCR, and inserted as an inverted repeat (IR) in a modified pUAST transformation vector, pUAST-R57 as described in Leulier *et al.* (2002). Transformation of *Drosophila* embryos was carried out in w^{1118} fly stocks. The *UAS-Mekk1-IR1* and *UAS-Mekk1-IR2* are independent insertions, both located on the second chromosome. The *da-GAL4* driver expresses *GAL4* strongly and ubiquitously (Leulier *et al.* 2002). *Hemolectin* (*hml*)-GAL4 is an hemocyte specific GAL4 driver (Goto *et al.* 2003) and *pumpless* (*ppl*)-GAL4 expresses GAL4 in the fat body (Colombani *et al.* 2003).

Infection and stress experiments

For septic injury and natural infection experiments, we used *Drosophila* adults, aged 2–4 days at 25 °C and reared under the same conditions as many environmental parameters cause considerable variability in *TotA* expression. Septic injury was performed by pricking the thorax of the flies with a needle previously dipped

into a concentrated mixed culture of *Escherichia coli* and *Micrococcus luteus*. Natural infection by the entomopathogenic fungus *Beauveria bassiana* was initiated by shaking anesthetized flies in a Petri dish containing a sporulating culture of the fungus (Lemaitre *et al.* 1997). Natural infections by *Erwinia carotovora 15* were performed by incubating *Drosophila* larvae in a mixture of crushed banana and bacteria (Basset *et al.* 2000). For survival experiments, flies were incubated at 25 °C after infection. For Northern and microarray analysis, flies were incubated at 25 °C and collected at specific times after infection.

Other stress inductions were performed as follows: for dehydration, flies were placed in an empty vial for 120 min; mechanical pressure was applied by squeezing flies for 1 h with a sponge plug without rupture of the cuticle; flies were heat-shocked during 3 h at 37 °C; osmotic stress was applied by placing flies on a vial containing 5 M NaCl food.

Northern blot analysis

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

Quantitative RT-PCR

For *TotA*, *upd3* and *rp49* mRNA quantification from whole animals, RNA was extracted using RNA Trizol™. cDNAs were synthesized from 1 μg of total RNA using SuperScript II (Invitrogen) and PCR was performed using dsDNA dye SYBR Green I (Roche Diagnostics). Primer pairs for *TotA* (forward 5′-GCA CCC AGG AAC TAC TTG ACA TCT-3′, and reverse 5′-GAC CTC CCT GAA TCG GAA CTC-3′), for *upd3* (forward 5′-GGC CCG TTT GGT TCT GTA GA-3′, and reverse 5′-GTA GAT TCT GCA GGA TCC TT-3′) and control *rp49* (forward 5′-GAC GCT TCA AGG GAC AGT ATC TG-3′, and reverse 5′-AAA CGC GGT TCT GCA TGA G-3′) were used to detect target gene transcripts. SYBR Green analysis was performed on a Lightcycler (Roche). All samples were analyzed in duplicate and the amount of mRNA detected was normalized to control *rp49* mRNA values. We used normalized data to quantify the relative levels of *TotA* mRNA according to cycling threshold analysis (ΔCt).

Analysis of mRNA expression using oligonucleotide arrays

Total RNA was extracted from 25 flies for each time point using Trizol reagent (GibcoBRL). Gene expression analysis was performed using the Affymetrix *Drosophila* GeneChip™, using the laboratory methods in the Affymetrix GeneChip expression manual. Briefly, double stranded cDNA was synthesized using 2 g of RNA. Biotin-labeled cRNA was synthesized using BioArray high yield RNA transcript labeling kit (Enzo) and 15 g of fragmented RNA were hybridized to each array. The arrays were washed using the EukGW2 protocol on the GeneChip Fluidics Station 400 series and scanned using the GeneArray scanner. Gene expression analysis was performed using multiple arrays, and multiple independent mRNA samples for each time point.

Data Analysis: Genes are represented on the DrosGenome1 chip by one or more transcripts, which in turn are represented by a probe set. Each probe set consisted of 14 pairs of perfect match (PM) and mismatch (MM) oligos. Data were collected at the transcript level, but for ease in the text, the data is referred to by gene. Intensity data for each feature on the array was calculated from the images generated by the GeneChip scanner using the GeneChip Microarray Suite. This intensity data was loaded into a MySQL database, where information on each of the features was also stored. The difference between the perfect match and mismatch oligos (probe pair) was calculated and the mean PM—MM intensity for each array was set to a constant value by linearly scaling array values. The mean intensity of individual probe pairs was calculated across all 34 arrays, and the $\log_2$ ratio of each value to this mean was stored. Next, all log ratios for each probe pair set (transcript) were averaged creating one measurement for each transcript on each array. The final dataset was generated by averaging data for each transcript on replicate arrays and subtracting the value of the uninfected sample from each measurement. We restricted our analysis to the 400 DIRGs already identified by De Gregorio *et al.* (2001). A threshold of 2 in two different time points was used to select the genes affected by the *Mekk1* mutation.

Oxidative stress resistance tests

We used 50 mL vials containing 1 mL of a solid medium composed of 1.3% low melting agarose, 1% sucrose and 10 mM paraquat. These compounds were incorporated at 45 °C to avoid loss of oxidative activity. Three-to five-day-old males were placed by groups of 30 in these vials and maintained at 26 °C. Dead flies were counted twice a day until the end of the experiment. For each experimental condition, at least three vials of 30 males were used for each genotype. In addition, to minimize genetic background effects, all the lines used in these experiments (with the exception of the $Mekk1^{Ur36}$ mutation, which is not associated to a visible marker) were previously out-crossed for at least four generations against a *w* Canton S reference line. For heat-shock rescue experiments, flies were heat-shocked 30 min at 37 °C before transfer to vials containing paraquat medium. To ensure a sustained expression of the transgene, subsequent 20 min 37 °C heat-shocks were performed on these flies every 24 h.

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