Thioester-Containing Protein-4 Regulates the *Drosophila* Immune Signaling and Function against the Pathogen *Photorhabdus*

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Key Words

Drosophila · Thioester-containing protein · *Photorhabdus* · Immune signaling · Melanization

Abstract

Despite important progress in identifying the molecules that participate in the immune response of *Drosophila melanogaster* to microbial infections, the involvement of thioester-containing proteins (TEPs) in the antibacterial immunity of the fly is not fully clarified. Previous studies mostly focused on identifying the function of TEP2, TEP3 and TEP6 molecules in the *D. melanogaster* immune system. Here, we investigated the role of TEP4 in the regulation and function of *D. melanogaster* host defense against 2 virulent pathogens from the genus *Photorhabdus*, i.e. the insect pathogenic bacterium *Photorhabdus luminescens* and the emerging human pathogen *P. asymbiotica*. We demonstrate that *Tep4* is strongly upregulated in adult flies following the injection of *Photorhabdus* bacteria. We also show that *Tep4* loss-of-function mutants are resistant to *P. luminescens* but not to *P. asymbiotica* infection. In addition, we find that inactivation of *Tep4* results in the upregulation of the Toll and Imd immune pathways, and the downregulation of the Jak/Stat and Jnk pathways upon *Photorhabdus* infection. We document that loss of *Tep4* promotes melanization and phenoloxidase

activity in the mutant flies infected with *Photorhabdus*. Together, these findings generate novel insights into the immune role of TEP4 as a regulator and effector of the *D. melanogaster* antibacterial immune response.

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Introduction

A key innate immune defense against bacterial infection includes the activation of the complex complement system that involves the action of complement proteins. These proteins contain an unstable thioester bond and belong to the family of thioester-containing proteins (TEPs). Most TEPs share a 4-amino-acid sequence (CGEQ) that allows covalent bond formation with microbial surfaces and promotes opsonization [1]. Their function involves the recognition and phagocytosis of microbes that ultimately lead to their elimination from the host [2].

TEPs emerged early in evolution and are present in a wide variety of organisms [3]. TEPs have been found to participate in the mosquito immune response to certain bacteria and protozoa parasites [4, 5]. For example, it was previously reported that the transcription of *Tep1* in the African malaria mosquito *Anopheles gambiae* (*aTep1*) is upregulated upon infection with *Escherichia coli* and

aTEP1 protein functions as an opsonin that promotes phagocytosis of *E. coli* and other Gram-negative bacteria [5]. It has also been shown that RNAi-mediated knock down of *aTep1* prevents melanization of *Plasmodium* parasites [6]. Although there is sufficient information on the function of mosquito TEPs against *Plasmodium* parasites and bacteria, there are only a few studies on the potential antimicrobial immune role of TEPs in the insect model, *Drosophila melanogaster* [1, 7, 8].

The *D. melanogaster* genome contains 6 *Tep* genes (*Tep1*–*Tep6*), of which *Tep5* is a pseudogene and *Tep6* or macroglobulin-complement related (*MCR*) lacks a functional thioester-binding site because of the substitution of cysteine residue by serine in the thioester motif [1]. TEP1–TEP4 proteins are constitutively expressed in plasmatocytes [1] whereas TEP6 has been shown to be a transmembrane protein that is required for septate junction formation [9]. Specifically, the *Tep4* gene is expressed in both larval stages as well as in adult flies [1]. Other than plasmatocytes, it is induced in the fat body of the head and ventral side of the abdominal epidermis and spermatozoon [1, 10, 11].

Few studies have examined the role of TEPs in the innate immune response of *D. melanogaster*. Previously, it has been shown that TEP2, TEP3 and TEP6 promote phagocytosis in *Drosophila* S2 cells after infection with the bacteria *E. coli* and *Staphylococcus aureus* or the fungus *Candida albicans*, respectively [8]. A recent study has also reported the high sensitivity of *D. melanogaster* *Tep3* mutant larvae to infection with the nematode parasite *Heterorhabditis bacteriophora* [12]. While previous studies focused mostly on TEP2, TEP3 and TEP6 molecules, the involvement of TEP4 in the antimicrobial immune response of the fly is largely unexplored. Whole-genome expression profiles have indicated that *Tep4* is differentially induced upon bacterial, fungal and parasitoid infection [1, 13, 14]. However, no changes were found in the survival response of *Tep4* mutant flies against infection with various bacterial and fungal pathogens [1]. It was concluded that the potential contribution of *Tep* genes in the *D. melanogaster* immune response could be better understood by using insect-specific pathogens, such as *Photorhabdus* bacteria.

Photorhabdus is a genus of entomopathogenic bacteria and a member of the family Enterobacteriaceae. In addition to being a highly virulent pathogen of insects, *Photorhabdus* maintains a mutualistic relationship with nematodes from the family Heterorhabditidae [15]. Here we have used two distinct species of *Photorhabdus* bacteria; *P. luminescens*, which is an obligate insect pathogen and

P. asymbiotica, which is able to infect both insects and humans [16]. The bacteria produce a wide range of toxins, virulence factors and hydrolytic enzymes [17, 18]. Important genomic differences between these two *Photorhabdus* species include the presence of a plasmid related to *pMT1* from *Yersinia pestis* together with novel pathogenicity islands in *P. asymbiotica* that converts this bacterium into a human pathogen. In addition, the presence of fewer diverse insecticidal genes than in *P. luminescens* allows *P. asymbiotica* to also persist as an insect pathogen [19, 20].

Here, we report the involvement of TEP4 in the innate immune response of the fly against *P. luminescens* and *P. asymbiotica*. We determine that transcription of *Tep4* gene plays an essential role in the regulation of immune signaling during *Photorhabdus* infection and that interference with *Tep4* gene transcription can affect the resistance and phenoloxidase (PO) activity in response to infection by these pathogens. These findings are important because they provide compelling evidence for the complexity and flexibility of the fly immune system.

Materials and Methods

Fly and Bacterial Strains

D. melanogaster fly strains *yw* and the P-element insertion mutant *tep4* (15936, Bloomington) were used for infections. Fly strains were maintained and amplified for experimentation with instant *Drosophila* media (Carolina Biological Supply) made in de-ionized water. All stocks were maintained at 25°C and a 12:12-hour light:dark photoperiod. Adult flies (7–10 days old) were used in all bacterial infection assays.

Bacterial strains *P. luminescens* subsp. *laumondii* (strain TT01), *P. asymbiotica* subsp. *asymbiotica* (strain ATCC 43949) and *E. coli* K12 were used for all fly infections. Bacterial cultures were prepared in sterile Luria-Bertani broth and grown for approximately 18–22 h at 30°C on a rotary shaker at 220 rpm. Bacterial cultures were pelleted down and were washed and resuspended in 1× sterile phosphate-buffered saline (PBS, Sigma Aldrich). Bacterial concentrations were adjusted to an optical density (OD, 600 nm) of 0.1 for *P. luminescens*, 0.25 for *P. asymbiotica* and 0.015 for *E. coli*, using a spectrophotometer (NanoDrop™ 2000c, Thermo Fisher Scientific).

Infection Assays and Survival Experiment

Flies were anesthetized with carbon dioxide and then injected in the thorax with 18.4 nl (100–300 CFU) of each bacterial suspension (i.e. *P. luminescens*, *P. asymbiotica* or *E. coli*) or PBS (septic injury control) using a Nanoject II apparatus (Drummond Scientific) equipped with glass capillaries prepared with a micropipette puller (Sutter Instruments). Following injection, flies were transferred to fresh vials with instant media at 25°C and survival was scored at 6-hour intervals and up to 48 h. Two replicates of 10 flies were used for each treatment and each assay was replicated 3 times.

Fig. 1. Transcript levels of *Tep4* in *yw* flies following *Photorhabdus* infection. Transcript levels of *Tep4* in *yw* flies at 6 and 18 h after injection with PBS, *E. coli* (Ec), *P. luminescens* (Pl) or *P. asymbiotica* (Pa) bacteria. Transcript levels are shown as relative abundance of transcripts normalized to *RpL32* and are presented as the ratio between uninfected flies versus bacteria or PBS injected flies (3 individuals/experimental condition). ** $p < 0.01$, **** $p < 0.0001$, significant differences. Bars show the means from 3 independent experiments and error bars represent standard deviations.

Bacterial Load

Four adult flies from each strain were injected with *P. luminescens*, *P. asymbiotica* or *E. coli*, and then frozen at 6 and 18 h after infection. DNA samples were extracted from the frozen flies using DNeasy blood and tissue kit (Qiagen). For bacterial load estimation, all DNA samples were adjusted to 500 ng. Quantitative PCRs were carried out in twin-tech, semiskirted, 96-well plates on a Mastercycler[®] ep realplex2. The cycling conditions for estimating the bacterial load were 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 15 s and an annealing step of 61°C for 15 s. All samples were run in technical duplicates and the experiments were repeated 3 times. Standard curves for each bacterium were used to estimate the bacterial load in infected flies as described before [21].

Gene Transcription

Three to four adult flies from each strain were injected with *P. luminescens*, *P. asymbiotica*, *E. coli* or PBS and frozen 6 and 18 h later. Total RNA was extracted using the PrepEase RNA spin kit (Affymetrix USB). Complementary DNA synthesis, quantitative RT-PCR (qRT-PCR) and analysis of the data were performed as previously described [22]. All primers used for the PCR assays are listed in online supplementary table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000450610). Data are presented as the ratio between uninfected flies versus bacteria- or PBS-injected flies.

Hemolymph Extraction, PO Activity and Melanization

For hemolymph collection, 20 flies were injected with bacteria or PBS as mentioned above, and PO activity was measured as de-

scribed previously [23]. Briefly, at 3 h after injection, the flies were placed on a 10- μ m spin column (Pierce, Thermo Fisher) containing 30 μ l of 2.5 $\times$ protease inhibitor (Sigma) and covered with five 4-mm glass beads (VWR). These were centrifuged at 4°C and 13,000 rpm for 20 min. Protein concentrations were then adjusted using a BCA test. A total volume of 40 μ l containing a mixture of 15 μ g of protein (diluted in 2.5 $\times$ protease inhibitor) with 5 mM CaCl₂ was added to 160 μ l of L-DOPA solution (15 mM in phosphate buffer, pH 6.6). After 36 min of incubation at 29°C in the dark, the PO activity (OD 492 nm) was measured for each sample against a blank control. Each experiment was performed in biological duplicate and repeated 3 times. Melanization spots on the site of injury were observed at 3 h after injection, using a Nikon SMZ18 stereomicroscope ($\times 10$ magnification) with Nikon EOS Rebel T3i camera.

Statistical Analysis

Statistics were performed using the GraphPad Prism6 software. Statistical analysis of data from survival experiments was conducted using the log-rank (Mantel-Cox) and χ^2 tests. Unpaired two-tailed Student's *t* test and one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons were used for analyzing bacterial load, gene expression data and PO activity results. $p < 0.05$ was considered statistically significant.

Results

Tep4 Is Induced by *Photorhabdus* Infection

We first examined the transcript levels of *Tep4* in the untreated flies of the reference strain (*yw*) and the loss-of-function *tep4* mutants. We found that transcript levels of *Tep4* were significantly reduced in the mutants compared to the background reference strain (online suppl. fig. S1a). We then investigated the transcriptional regulation of *Tep4* in background flies injected by either *Photorhabdus* sp., *E. coli* (nonpathogenic control) or PBS (septic injury control) at 6 and 18 h after injection. We observed that *Tep4* was upregulated at 6 h after infection with *E. coli* and *P. asymbiotica* whereas after 18 h, *Tep4* was induced by the *Photorhabdus* bacteria only (fig. 1). We also noticed no changes in *Tep4* gene transcript levels in *tep4* mutants upon bacterial infection or in the uninfected controls at any of the time points tested (online suppl. fig. S1b). These results show that infection of *D. melanogaster* with the insect-specific pathogen *P. luminescens* or the related human pathogen *P. asymbiotica* results in significant upregulation of *Tep4* in the adult fly.

Tep4 Mutants Are Resistant to *P. luminescens* Infection

To test whether inactivation of *Tep4* affects the survival ability of the fly against infection by *Photorhabdus* pathogens, we tested the survival response of *tep4* loss-of-

Fig. 2. *Tep4* mutants are resistant to *P. luminescens* during the initial phases of infection. Survival of loss-of-function *tep4* mutants and control flies (*yw*) after intrathoracic injection with *P. luminescens* (*Pl*) (**a**) or *P. asymbiotica* (*Pa*) (**b**) (20 flies/experimental condition). Injections with PBS served as injury control. Survival was monitored for 48 h at 6-hour intervals and the black dotted line represents 50% survival. CFU of *P. luminescens* (**a**) or *P. asym-*

biotica (**b**) at 6 and 18 h after injection were determined by quantitative PCR of *mcf-1* (*P. luminescens*) and *tccC3* (*P. asymbiotica*) in *tep4* mutants and their background control *yw* flies (5 individuals/experimental condition). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant differences. The means from 3 independent experiments are shown and error bars represent standard errors (survival) and standard deviation (bacterial load).

function mutants and their background controls to infection by *P. luminescens* and *P. asymbiotica*. We found that *tep4* mutants and control flies succumbed within 36 h of *P. luminescens* infection and within 48 h of *P. asymbiotica* infection. We noticed that after 24 h, *tep4* mutants had survived significantly better (67% were alive) than the control flies (0% were alive) (fig. 2a). Likewise, 85% of *tep4* mutants and 65% of the controls were alive 24 h after *P. asymbiotica* infection (fig. 2b). Thirty-six hours after infection with *P. asymbiotica*, 37% of *tep4* mutant flies were alive versus 8% of the controls (fig. 2b). We further

observed that injection with nonpathogenic *E. coli* or sterile PBS did not affect the survival of *tep4* mutant flies or their background controls (online suppl. fig. S2a). These results indicate that inactivation of *Tep4* provides a survival advantage to *D. melanogaster* flies in response to infection with *P. luminescens* and *P. asymbiotica* pathogenic bacteria.

To investigate whether increased survival of *tep4* loss-of-function mutants to *Photorhabdus* infection is due to the decreased persistence of the bacteria in the infected flies, we estimated bacterial load during the course of the

infection. For this, we evaluated the number of CFU by quantitative PCR by amplifying the genes *mcf-1* and *tccC3* in *P. luminescens* and *P. asymbiotica* [24, 25], respectively, at 6 and 18 h after infection. Previous studies have shown that the levels of virulence factors produced by *Photorhabdus* do not change during insect infection [26]. We observed that *tep4* mutants contained 12 times fewer numbers of *P. luminescens* CFU compared to the background controls at 18 h after infection (fig. 2a), but there were no significant differences in *P. asymbiotica* CFU between the 2 fly strains (fig. 2b). Also, *tep4* mutants and their controls contained similar numbers of *E. coli* cells (online suppl. fig. S2b). These results indicate that deficiency in *Tep4* confers resistance to *P. luminescens* infection only.

Functional *Tep4* Is Essential for Immune Signaling Activation upon *Photorhabdus* Infection

The hallmark of the *D. melanogaster* host defense is the definition of 2 major signaling pathways, Toll and Immune deficiency (Imd). The Toll pathway is reminiscent of the IL1/TLR4 Myd-88 dependent pathway whereas the Imd pathway presents striking similarities with the TNFR and TLR4 MyD88-independent pathways [27, 28]. These pathways activate different members of the NF- κ B family of transcription factors, and induce the expression of several target genes including those encoding antimicrobial peptides (AMP) [29, 30]. The Toll pathway is induced by Lys-type peptidoglycan present on most Gram-positive bacteria, while the Imd pathway is triggered by diaminopimelic acid-type peptidoglycan that composes the cell wall of Gram-negative and some Gram-positive bacteria [31]. The Janus kinase/signal transducer and activator of transcription (Jak/Stat) and c-Jun N-terminal kinase (Jnk) signaling pathways can also act in either competing or cooperative modes to regulate immune effector genes in the fly [32, 33]. To investigate whether the increased survival of *Tep4* mutant flies to *Photorhabdus* infection could also be due to increased immune signaling activation, we examined the transcriptional activation of Toll, Imd, JAK/STAT and JNK immune pathways in *tep4* loss-of-function mutant flies infected with the bacteria. We first estimated the transcript levels of the AMP *Defensin* as a bacterial-specific readout of the Toll pathway [29]. We have previously shown that infection of wild-type flies with *Photorhabdus* induces low levels of *Defensin* [21, 22]. Therefore, we asked whether *Tep4* inactivation can alter the transcript levels of *Defensin* in response to *Photorhabdus*. We observed that *Defensin* was slightly upregulated in *yw* flies at 6 h after infection with any of the 3 bacteria (fig. 3a; online suppl. fig. S3a). Interestingly,

there was an early (at 6 h) transcriptional activation of *Defensin* in *tep4* mutants upon infection with *P. luminescens* or *P. asymbiotica*, but not with nonpathogenic *E. coli* (fig. 3a; online suppl. fig. S3a). Then, we evaluated Imd pathway activation by estimating the transcript levels of the *Cecropin-A1* (AMP) gene in infected flies [30, 34]. We observed significantly higher transcript levels of *Cecropin-A1* in *tep4* mutants than in their *yw* background controls following infection for 18 h with *Photorhabdus* (fig. 3b). These results indicate that inactivation of *Tep4* results in the activation of Toll and Imd signaling in response to *Photorhabdus* infection.

We then examined the activation of Jak/Stat and Jnk signaling pathways in *tep4* mutant and their background controls, by assessing the transcript levels of *Turandot-M* (*Tot-M*) and *Puckered* (*Puc*) [35, 36]. We noticed that *Tot-M* was upregulated in *yw* flies at 6 h after infection with either *Photorhabdus* species or *E. coli* (fig. 3c; online suppl. fig. S3c). We then observed that in *tep4* mutant flies, *Tot-M* was not upregulated at any time point after bacterial infection compared to background controls (fig. 3c; online suppl. fig. S3c). Also, *Puc* was significantly upregulated in control flies at 18 h after infection with *Photorhabdus* bacteria, but not with *E. coli* (fig. 3d; online suppl. fig. S3d). Strikingly, transcript levels of *Puc* in *tep4* mutants were significantly lower than in control flies upon infection with *P. asymbiotica* or *P. luminescens* (fig. 3d). These results indicate that activity of *Tep4* is important for the regulation of Jak/Stat and Jnk signaling in *D. melanogaster* adult flies upon infection with the pathogen *Photorhabdus*.

Inactivation of *Tep4* Increases the Melanization and PO Response to *Photorhabdus* Infection

We also asked whether inactivation of *Tep4* affects PO activity and the melanization response, which form rapid immune reactions of the fly against microbial intruders [23]. Since the formation of a black spot around the injection site represents the output of the melanization reaction [37], here we first examined the in vivo melanization in *tep4* mutants and their control flies by visually inspecting the wound site at 3 h after injection with *Photorhabdus* sp., *E. coli* or PBS. We noticed the development of dark melanin spots in *tep4* mutants in response to bacteria, but *yw* flies developed fainter spots after challenge with bacteria (fig. 4a; online suppl. fig. S4a).

To quantify the level of PO enzyme activity in infected and uninfected *tep4* mutants and their background controls, we extracted hemolymph plasma from the flies at 3 h after injection with bacteria or buffer alone. We found

Fig. 3. *Tep4* modulates the activation of immune signaling in response to *Photorhabdus*. Transcript levels for *Defensin* (Toll pathway, **a**), *Cecropin-A1* (Imd pathway, **b**), *Tot-M* (Jak/Stat pathway, **c**) and *Puckered* (Jnk pathway, **d**) in *tep4* mutant and background control flies (*yw*) at 0, 6 and 18 h after infection with *P. luminescens* or *P. asymbiotica* ($n = 3$ individuals per experimental condition).

Gene transcript levels are shown as relative abundance of transcripts normalized to *RpL32* and expressed as a ratio compared to flies injected with sterile PBS (negative control). Values represent the means from 3 biological replicates, and error bars represent standard deviations. * $p < 0.05$, ** $p < 0.01$, significant differences.

Fig. 4. Melanization response and PO activity are elevated in *tep4* mutants upon *Photorhabdus* infection. **a** Melanization of the wound site in *tep4* loss-of-function mutant flies and their background control strain (*yw*) is shown 3 h after injection with PBS, *P. luminescens* or *P. asymbiotica*. Arrows indicate the site of injury. $\times 10$. **b** PO activity in the hemolymph plasma of *tep4* mutants and control flies (*yw*) 3 h after injection with PBS, *P. luminescens* (Pl) or *P. asymbiotica* (Pa) ($n = 20$ flies), as measured by the OD at 492 nm after incubation with L-DOPA. Values represent the means from 3 biological replicates and error bars represent standard deviations. *** $p < 0.001$, significant differences.

no significant changes in PO activity between *tep4* mutant flies and *yw* controls injected with PBS (fig. 4b). However, *tep4* mutants infected by either *Photorhabdus* sp. or *E. coli* displayed significantly higher PO activity levels than *yw* flies (fig. 4b; online suppl. fig. S4b). These results imply that the absence of functional *Tep4* in *D. melanogaster* adult flies promotes PO activity and the melanization response against infection with pathogenic *Photorhabdus* or nonpathogenic *E. coli* bacteria.

Discussion

Significant advances have been made recently in the field of *Drosophila* immunity; however, our understanding of the role of TEP molecules in the fly immune response remains incomplete [1, 30]. Here, we have investigated the participation of *Tep4* in the immune activity of *D. melanogaster* against two species of the virulent pathogen *Photorhabdus*. Although *D. melanogaster* has

not been shown thus far to be a natural host for *Phototrhabdus*, we and others have recently started to exploit the powerful tools of the fly to identify the molecules potentially involved in the interaction between the fly immune system and pathogenic microbes [22, 38, 39]. *Phototrhabdus* bacteria express multiple pathogenicity factors with different specificities against a diverse range of insect species and tissues [40, 41], so we hypothesized that the response of *D. melanogaster* to *Phototrhabdus* bacteria would be sufficiently different from other classes of bacteria that have been studied in this system [30].

Previous transcriptomic analyses have shown that *Tep2* and *Tep4* are upregulated in whole flies following infection with a mix of *E. coli* and *Micrococcus luteus* bacteria [1, 42, 43]. Here, we showed that *Tep4* is transcriptionally activated by *P. luminescens* and *P. asymbiotica* infection, which implies that *D. melanogaster* responds differently to specific pathogens. Our finding that *Tep4* is upregulated upon *E. coli* challenge is in accordance with the previous studies [1, 42]. The upregulation of *Tep4* in *D. melanogaster* by *Phototrhabdus* bacteria prompted us to speculate that certain TEP molecules might play an important role in the interaction between the fly immune response and *Phototrhabdus*. The outcome of this interaction could involve changes in the sensitivity of the flies upon infection with these pathogens due to altered immune signaling or function. We have found that loss of *Tep4* delays the mortality of mutant flies during the initial and intermediate stages of infection with *P. luminescens* or *P. asymbiotica*. Consequently, inactivation of *Tep4* partially promotes the survival of the fly against *Phototrhabdus*; however, the protective effect is not complete because the mutants eventually succumb to infection with the pathogens. Lack of changes in the sensitivity of *tep4* mutants to nonpathogenic *E. coli* infection indicates that the protective effect is predominantly or exclusively conferred to the pathogens. Similar to our findings, a previous study also reported increased survival of hemizygous *tep4* mutants towards bacterial infection [1]; however, another study has shown *tep4* mutants to be sensitive to infection with the bacterium *Porphyromonas gingivalis* [7].

Given that *Phototrhabdus* is not a natural pathogen of *D. melanogaster*, the positive effect of *Tep4* gene inactivation on the survival of the mutant flies is probably specific to these pathogens as well as to as-of-yet unidentified natural pathogens of *D. melanogaster* with a mode of action similar to *Phototrhabdus* [17, 20]. Such natural pathogens may employ distinct strategies to interact with the *D. melanogaster* immune system as a result of antagonis-

tic coevolution with the fly. This assumption is supported by a previous study which examined the molecular evolution of innate immune system components in *D. melanogaster*, and proposed that certain *Tep* genes might have specific functions against distinct pathogens as a result of their rapid evolution due to strong positive selection forces that might act on these molecules [44]. An alternative explanation for the increased survival of *tep4* mutants could be that upregulation of *Tep4* in flies infected with certain pathogens, such as *Phototrhabdus*, result in inflammatory responses that severely affect the survival of the flies during the first few hours of infection and eventually lead to insect death. Lack of changes in the *P. asymbiotica* burden between *tep4* mutants and control flies imply the involvement of other immune mechanisms that could promote survival upon infection with this pathogen.

There is a previous indication that *Phototrhabdus* can interact with AMP gene expression in the fly [22], and that ligands produced by hemocytes could induce immune signaling in certain tissues (e.g. the fat body) that could, in turn, affect the activation of *Tep* genes [32]. The effect of *Tep4* gene inactivation on the increased survival of mutant flies with *Phototrhabdus* infection formed the basis for testing whether *Tep4* activity plays a regulatory role in the *D. melanogaster* immune system. For this, we examined whether a loss of *Tep4* can affect the outcome of immune signaling in response to *Phototrhabdus*. Unexpectedly, Toll and Imd pathway activation was consistently affected in the *tep4* mutant flies following *P. luminescens* or *P. asymbiotica* infection. Similarly, Jak/Stat and Jnk pathways were significantly impaired in the *tep4* mutants upon *Phototrhabdus* infection. Our results strongly indicate that normal transcription of *Tep4* is required for the efficient activation of immune signaling in *D. melanogaster* flies responding to *Phototrhabdus*. Since TEP4 is a secreted molecule [45], it may interact with the hemocytes and fat-body cells in a distinct manner when *Phototrhabdus* bacteria are present in the fly hemocoel. Such interactions could then lead to changes in the regulation of genes that are induced in these tissues and the expression of which is controlled by specific immune signaling pathways. Therefore changes in immune signaling in *Phototrhabdus*-infected flies, in which *Tep4* gene expression is compromised, potentially lead to the production of molecules (presumably AMP) that provide protection to the pathogens during the early or intermediate stages of infection.

The proPO system is a rapid and efficient antimicrobial immune reaction that links humoral and cellular im-

Fig. 5. Proposed model for the participation of TEP4 in the immune response of *D. melanogaster* against the pathogen *Photorhabdus*. 1. *Tep4* is upregulated upon *Photorhabdus* infection. 2. Upregulation of *Tep4* reduces Toll and Imd signaling in response to *Photorhabdus* infection. 3. Upregulation of *Tep4* is required for the activation of Jak/Stat and Jnk signaling against *Photorhabdus*. 4. Regulation of immune signaling pathways by *Tep4* induction leads to differential induction of AMP-encoding genes and stress-related genes following infection with *Photorhabdus*. 5. During the early stages of *Photorhabdus* infection, upregulation of *Tep4* decreases the PO activity and melanization response. 6. Decrease in the activity of PO/melanization and interference with innate immune signaling alters the survival response of adult flies to *Photorhabdus* infection.

munity [46]. Previously, it has been proposed that during infection, TEP molecules in the hemolymph can interact with the fat body (and possibly with other tissues) to activate signaling pathways that regulate immune functions against the invading microbes [45]. Therefore, we tested whether infection with *Photorhabdus* could influence PO activity in the background control flies and whether inactivation of *Tep4* can further affect PO activity in the mutants infected with the pathogens. We were not surprised to find that *Photorhabdus* infection reduces PO activity in the hemolymph as well as the melanization response because both *P. luminescens* and *P. asymbiotica* employ mechanisms to interfere with the activation of the insect

proPO cascade [47–50]. Our results further reveal high levels of PO in *tep4* mutants infected with either *Photorhabdus* pathogen. Because melanization and activation of the proPO system are rapid immune responses, these results could also explain the increased persistence of *Photorhabdus* bacteria in *tep4* mutant flies over time. We suspect that high PO activity in *tep4* mutant flies reduces bacterial load and, as a result, improves survival during the early phase of infection. Furthermore, these findings show, for the first time, an apparent link between *tep4* transcriptional gene expression and PO activity. Interestingly, this effect is not restricted only to *Photorhabdus* infections, but also to infections with nonpathogenic

E. coli bacteria. This is an important finding because it shows that the proPO system in insects can be regulated by pathogens in order to evade the host immune system, but also by host molecules that can markedly affect immune signaling and microbial resistance and consequently influence the outcome of the infection.

Taken together, we have shown that *Tep4* can modulate the immune response of *D. melanogaster* adult flies against infection with the virulent pathogens *P. luminescens* and *P. asymbiotica*. This can be attributed to its critical role as a regulator of immune signaling and function in response to these pathogens (fig. 5). It is currently unknown whether the expression of TEPs in the fly can be activated by proteases produced by pathogenic microbes or by the host [44]. Given that *Photorhabdus* bacteria secrete a battery of proteases [51], our future efforts will focus on exploring their association with *Tep4* immune activity and the participation of the latter in cellular immune function of the fly against *Photorhabdus* and their cognate nematode partners [15]. Of note, certain *tep* fly

mutants were recently shown to be sensitive to infection by entomopathogenic nematodes when compared to background controls [12]. We expect that such studies will unravel the regulatory role and specific function of TEP molecules in the antipathogen immune response of the fly and probably that of other invertebrate animals.

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Disclosure Statement

The authors declare no competing interests.

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