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RESEARCH PAPER

The distinct function of *Tep2* and *Tep6* in the immune defense of *Drosophila melanogaster* against the pathogen *Photorhabdus*

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ABSTRACT

Previous and recent investigations on the innate immune response of *Drosophila* have identified certain mechanisms that promote pathogen elimination. However, the function of Thioester-containing proteins (TEPs) in the fly still remains elusive. Recently we have shown the contribution of TEP4 in the antibacterial immune defense of *Drosophila* against non-pathogenic *E. coli*, and the pathogens *Photorhabdus luminescens* and *P. asymbiotica*. Here we studied the function of *Tep* genes in both humoral and cellular immunity upon *E. coli* and *Photorhabdus* infection. We found that while *Tep2* is induced after *Photorhabdus* and *E. coli* infection; *Tep6* is induced by *P. asymbiotica* only. Moreover, functional ablation of hemocytes results in significantly low transcript levels of *Tep2* and *Tep6* in response to *Photorhabdus*. We show that *Tep2* and *Tep6* loss-of-function mutants have prolonged survival against *P. asymbiotica, Tep6* mutants survive better the infection of *P. luminescens*, and both *tep* mutants are resistant to *E. coli* and *Photorhabdus*. We also find a distinct pattern of immune signaling pathway induction in *E. coli* or *Photorhabdus* infected *Tep2* and *Tep6* mutants. We further show that *Tep2* and *Tep6* participate in the activation of hemocytes in *Drosophila* responding to *Photorhabdus*. Finally, inactivation of *Tep2* or *Tep6* affects phagocytosis and melanization in flies infected with *Photorhabdus*. Our results indicate that distinct *Tep* genes might be involved in different yet crucial functions in the *Drosophila* antibacterial immune response.

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Introduction

Drosophila melanogaster has served as an excellent model system to study innate immune defense mechanisms against microbial infections.[1] To detect different types of pathogens, the fly uses specific pattern recognition receptors such as peptidoglycan recognition receptors, Gram-negative binding proteins, scavenger receptors or Thioester-containing proteins (TEPs).[2] Most TEPs contain a highly reactive thioester motif that covalently binds to the microbial surfaces and leads to their elimination from the host. Although a vast amount of information is available on various pattern recognition receptors in *Drosophila*, the specific function of TEPs is still not entirely understood. However, their immune role is widely studied in the mosquitoes *Anopheles gambiae* and *Aedes aegypti*, and in vertebrates.[3-6] The *Anopheles* TEP1 is involved in the process of phagocytosis of *Escherichia coli* and *Staphylococcus aureus*, as well as in the melanization of *Plasmodium* parasites.[7] Similarly, *A. aegypti* macroglobulin complement-related (MCR) factor participates in fighting off flavivirus infection.[5]

Previous studies have shown that *Drosophila Tep1, Tep2, Tep3* and *Tep4* genes are induced upon certain bacterial, fungal, parasitoid and parasitic challenges.[8-10] Moreover, an in-vitro study has shown that phagocytosis of *E. coli* and *S. aureus* bacteria is regulated by TEP2, TEP3 and phagocytosis of *Candida albicans* spores by TEP6.[11] Recently we have shown that *Tep4* modulates the activation of Toll and IMD immune signaling in *Drosophila* flies responding to 2 species of the potent pathogen *Photorhabdus*.[12] We further reported that inactivation of *Tep4* leads to increased phenoloxidase and melanization activity upon *Photorhabdus* bacteria, and these effects alter the survival response of the flies to these pathogens.

The *Photorhabdus* genus contains bacteria that are highly virulent insect or human pathogens, which live in a mutualistic relationship with Heterorhabtidid nematodes.[13] The

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bacteria use distinct defense strategies that allow them to surpass the host immune responses. For example, a toxin secreted by *P. luminescens* has been shown to target a large number of insect hemolymph proteins encoding molecules that are involved in immune recognition, immune signaling and regulation of the coagulation cascade.[14] In addition, *Photorhabdus* can subvert cellular immune responses by secreting toxins or virulence factors that induce freezing or apoptosis of insect hemocytes.[15,16] Other studies have also revealed that *Photorhabdus* is able to interfere with the insect prophenoloxidase cascade.[17-19] *Photorhabdus* bacteria are closely related to many mammalian pathogens such as *Yersenia pestis, E. coli* and *Salmonella*.[20] Hence, results from studies on the pathogenicity of *Photorhabdus* in the context of host immune activity can be extrapolated to other pathogens of agricultural or medical importance.

To further our understanding on the immune role of *Drosophila* TEPs in the host defense against the pathogen *Photorhabdus*, here we have investigated the participation of *Tep* genes in the fly humoral and cellular antibacterial immune response. Using *tep* mutant flies together with gene expression assays and functional immune tests, we have shown that *Tep2* and *Tep6* are probably involved and act distinctly in the activation and regulation of immune signaling pathways, phagocytosis and phenoloxidase responses in the fly against the pathogen *Photorhabdus*.

Results

Tep genes are induced in *Drosophila* upon *Photorhabdus* challenge

Previously we have shown that *Tep4* is transcriptionally activated upon *E. coli or Photorhabdus* infection. Therefore, we first examined whether other *Tep* genes (*Tep1–3* and *Tep6*) have altered expression in the background fly strain (w^{1118}) upon infection with these bacteria. We found that certain *Tep* genes (*Tep1, Tep2* and *Tep6*) were upregulated at 6 and 18 hpi by mostly *P. luminescens* and *P. asymbiotica* infection (Fig. 1). Only *Tep2* was induced in flies infected with non-pathogenic *E. coli* bacteria (Fig. 1A) at 6 hpi. In particular, there was a significant induction of *Tep1* and *Tep2* genes in *P. luminescens* infected flies at 6 hpi (Fig. 1A), and *Tep1, Tep2* and *Tep6* genes in *P. asymbiotica* infected flies at 18 hpi (Fig. 1A–B). While *Tep2* was mainly upregulated by *P. luminescens* and *P. asymbiotica* at both time points, *Tep1* was significantly induced at higher levels by *P. luminescens* only at the 6 h time-point (Fig. 1A–B). These results show that infection of *D. melanogaster* with the insect-specific pathogen *P. luminescens* and the related human pathogen *P. asymbiotica* as well as *E. coli* results in

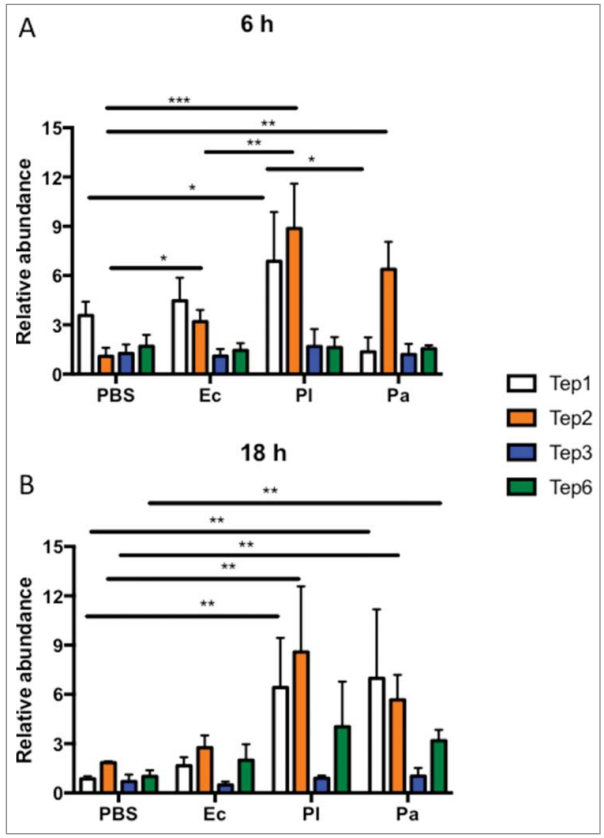

Figure 1. *Tep1, Tep2* and *Tep6* genes are upregulated in *D. melanogaster* flies by *Photorhabdus* infection. Transcript levels of *Tep1, Tep2, Tep3* and *Tep6* genes are shown in w^{1118} flies (n = 3–5) after (A) 6 and (B) 18 hpi with 1XPBS (septic injury control), *E. coli* ($_{Ec}$), *P. luminescens* (Pl) and *P. asymbiotica* (Pa). Gene transcript levels are shown as relative abundance of transcripts normalized to *RpL32* and expressed as a ratio compared with untreated flies (negative control). Significant differences are shown with asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Bars show the means from 3 independent experiments and error bars represent standard deviation.

significant induction of certain TEP coding genes in the adult fly.

Drosophila tep mutants have increased survival during the early and mid stages of *Photorhabdus* infection

To examine the function of the *Tep* induced genes in the immune response of *Drosophila*, we first performed survival analysis of the infected mutant flies. We tested the survival response of *Tep2* and *Tep6* loss-of-function mutants and their background control to infection by the 2 *Photorhabdus* pathogens and the non-pathogenic *E. coli*. We excluded *tep1* mutants because *Tep1* and *Tep2* mRNAs were expressed at similar levels (Fig. 1A–B) and according to a previous phylogenetic analysis, *Tep1* and *Tep2* are likely to act redundantly (Bou Aoun

et al, 2011). Moreover, we omitted *tep3* mutants as we did not observe any changes in the mRNA levels of *Tep3* (Fig. 1). To ascertain that we used loss-of-function *tep* mutants, we estimated the mRNA levels of *Tep2* and *Tep6* in the *tep2* and *Tep6* mutant flies injected with PBS, *E. coli* or *Photorhabdus* bacteria (Fig. S1 A-B).

Both *Tep2* and *Tep6* strains died within 36 h after *P. luminescens* infection and within 48 h after *P. asymbiotica* infection. We also observed that *Tep2* flies died similarly to their background controls when infected with *P. luminescens* but survived significantly longer when infected with *P. asymbiotica* (Fig. 2A–B). We found that at 36 hpi with *P. asymbiotica*, 48% of *Tep2* were alive compared with their background controls (~1%). However, there were 75% of *Tep6* mutant flies alive compared with 14% of controls at 24 hpi with *P. luminescens* whereas 47% of *Tep6* mutants were alive at 36 h post *P. asymbiotica* infection compared with controls (~2%) (Fig. 2B). We further found that injection with non-pathogenic *E. coli* or sterile PBS did not affect the survival of *tep* mutant flies and their background controls (Fig. S3A-B). These results indicate that loss-of-function mutations in *Tep6* provide a survival advantage to *D. melanogaster* in response to infection with *P. luminescens*, while loss-of-function mutations in *Tep2* and *Tep6* promote the survival of flies against infection with *P. asymbiotica*.

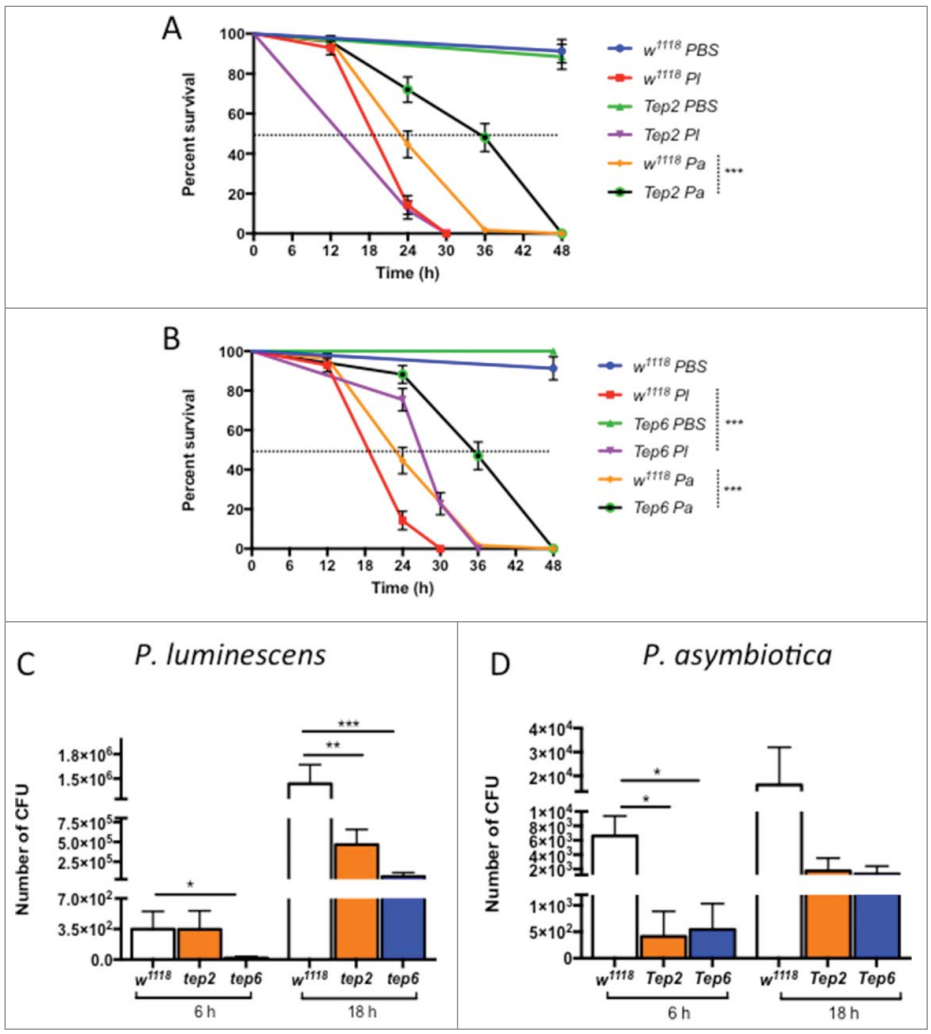

Figure 2. Survival and bacterial load analysis for *tep2* and *tep6* mutants after *Photorhabdus* infection. Survival curves for loss-of-function (A) *tep2* mutants and (B) *tep6* mutants with w^{1118} (background control flies) are shown. Flies (n = 20) were injected in the thorax by microinjection with 1XPBS (septic injury control), *P. luminescens* (Pl) or *P. asymbiotica* (Pa). Survival was monitored at 6 h intervals for 48 h. The black dotted line represents 50% survival. Colony forming units (CFU) of (C) *P. luminescens* and (D) *P. asymbiotica* are shown in *tep2, tep6* and control flies (n = 5 per experimental condition) after 6 and 18 hpi. CFU were quantified through quantitative PCR of *makes caterpillars floppy* (*mcf-1*) in *P. luminescens* and the insecticidal toxin complex protein gene (*tccC3*) in *P. asymbiotica*. Significant differences are indicated with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The means from 3 independent experiments are shown and error bars represent standard errors (survival) and standard deviation (bacterial load).

To understand the basis for the increased survival of the *tep* infected mutant flies, we investigated the bacterial persistence at 2 time-points post infection. To estimate bacterial load, we evaluated the number of colony forming units (CFU) at 6 and 18 hpi. We noticed that although there were no significant differences in survival between *Tep2* mutants and their controls, there were 3-times fewer *P. luminescens* CFU in the mutant flies than in w^{1118} individuals at 18 hpi (Fig. 2C). Similarly, *Tep6* mutants had 18-times and 22-times less *P. luminescens* CFU than the control flies at 6 and 18 hpi, respectively (Fig. 2C). In the case of *P. asymbiotica* infections, there were 3.5-times and 8-times fewer CFU in *Tep2* and *Tep6* mutants compared with w^{1118} flies at 6 h only (Fig. 2D). Additionally, infections with non-pathogenic *E. coli* resulted in significantly lower numbers of CFU in *Tep2* and *Tep6* mutants compared with w^{1118} controls at both time-points post infection (Fig. S2C). Interestingly, *Tep2* mutants contained 20-times more *E. coli* cells than *Tep6* flies (Fig. S2C). These results show that deficiencies in *Tep2* and *Tep6* genes confer resistance to *P. luminescens, P. asymbiotica* and *E. coli*.

Function of *Tep2 and Tep6* genes is essential for immune signaling pathway regulation in *Drosophila*

To explore the increased resistance of *Tep2* and *Tep6* mutants toward *Photorhabdus* and *E. coli*, we examined the transcriptional activation of Toll, Imd, JAK/STAT and JNK immune pathways in loss-of-function *Tep2* and *Tep6* mutant flies infected with these bacteria. We first tested at the activation of Toll pathway by evaluating the transcript levels of the AMP *Defensin*, which is a bacterial specific AMP.[21] In addition, we have recently found low to moderate transcript levels of *Defensin* in wild-type flies infected with *Photorhabdus*.[22,23] Here we asked whether flies with inactivated *Tep2* or *Tep6* have altered *Defensin* transcript levels upon infection with the pathogens. We found that *Defensin* was strongly induced in w^{1118} flies by either *Photorhabdus* species at 6 hpi but only by *P. luminescens* at 18 hpi (Fig. 3A–B). We further found that *Defensin* mRNA levels were significantly higher in *Tep6* mutant flies compared with their background controls at 6 hpi with *P. luminescens*, and at both 6 and 18 hpi upon infection with *P. asymbiotica* (Fig. 3A–B). We observed significant upregulation in the mRNA levels of *Defensin* in *Tep2* mutants at 6 and 18 hpi with *P. luminescens* compared with *Tep2* mutant flies injected with PBS (Fig. 3A–B). This indicates that *Tep6* but not *Tep2* gene activity is required in the induction of Toll pathway.

To evaluate Imd pathway activation, we estimated the transcript levels of the AMP-encoding gene *Diptericin* in infected flies (Fig. 3C–D).[24] We observed that *Diptericin* mRNA levels were significantly induced in the w^{1118} flies by *Photorhabdus* and *E. coli* bacteria at 6 and 18 hpi, as well as in PBS injected flies at 6 hpi (Fig. 3C–D). Moreover, upregulation of *Diptericin* was significantly higher in w^{1118} flies infected with *P. asymbiotica* compared with *E. coli* infected flies of the same strain at 18 hpi (Fig. 3D). Interestingly, we found that transcript levels of *Diptericin* were consistently lower in *Tep2* mutants than in w^{1118} flies at both time points (Fig. 3C–D). In addition, there were no differences in *Diptericin* mRNA levels between *Tep6* mutants and w^{1118} background controls (Fig 3C–D). These results indicate that *Tep2* regulates Imd signaling in *D. melanogaster* adult flies in the context of *Photorhabdus* infection or response to wounding.

To analyze JAK/STAT and JNK signaling activation in *tep* mutants and control flies, we assessed the transcript levels of *Turandot-M* (*Tot-M*) and *Puckered* (*Puc*).[22,23,25,26] We first observed that *Tot-M* was significantly upregulated in w^{1118} flies at 18 hpi with *P. luminescens* than flies injected with other bacteria or PBS (Fig. 3E–F). The *Tot-M* mRNA levels were significantly low in w^{1118} flies infected with *P. asymbiotica* than *P. luminescens* or PBS injected flies at 6hpi (Fig. 3E). We found that *Tep2* mutants have significantly reduced *Tot-M* mRNA levels than the w^{1118} flies injected with any of the bacteria at both time points (Fig. 3E–F). We also observed that in *Tep2* mutant flies, *Tot-M* was slightly upregulated only at 6 hpi with *P. luminescens* compared with other treatments, but this induction was significantly lower compared with background flies infected by *P. luminescens* (Fig. 3E). We further noticed that *Tot-M* was significantly upregulated in *Tep6* mutants at 6 hpi with *Photorhabdus* bacteria and at 18 hpi with *E. coli* in relation to control flies (Fig 3E–F). We found that *Puc* mRNA levels were significantly lower in *tep* mutants compared with the control flies injected with *P. asymbiotica* at 18 hpi only (Fig. 3G–H). These results indicate that *Tep2* is required for full JAK/STAT pathway induction in the presence of certain bacterial infections of adult fruit flies. In addition, *Tep2* and *Tep6* gene activity is required for JNK signaling in *D. melanogaster* adult flies upon infection with *P. asymbiotica* during the late stages of infection.

Functional hemocytes in *Drosophila* constitute a source of *Tep2* and *Tep6* transcription

Because TEPs are secreted proteins and they are expressed in larval plasmatocytes,[10] we examined whether changes in the function of hemocytes can affect

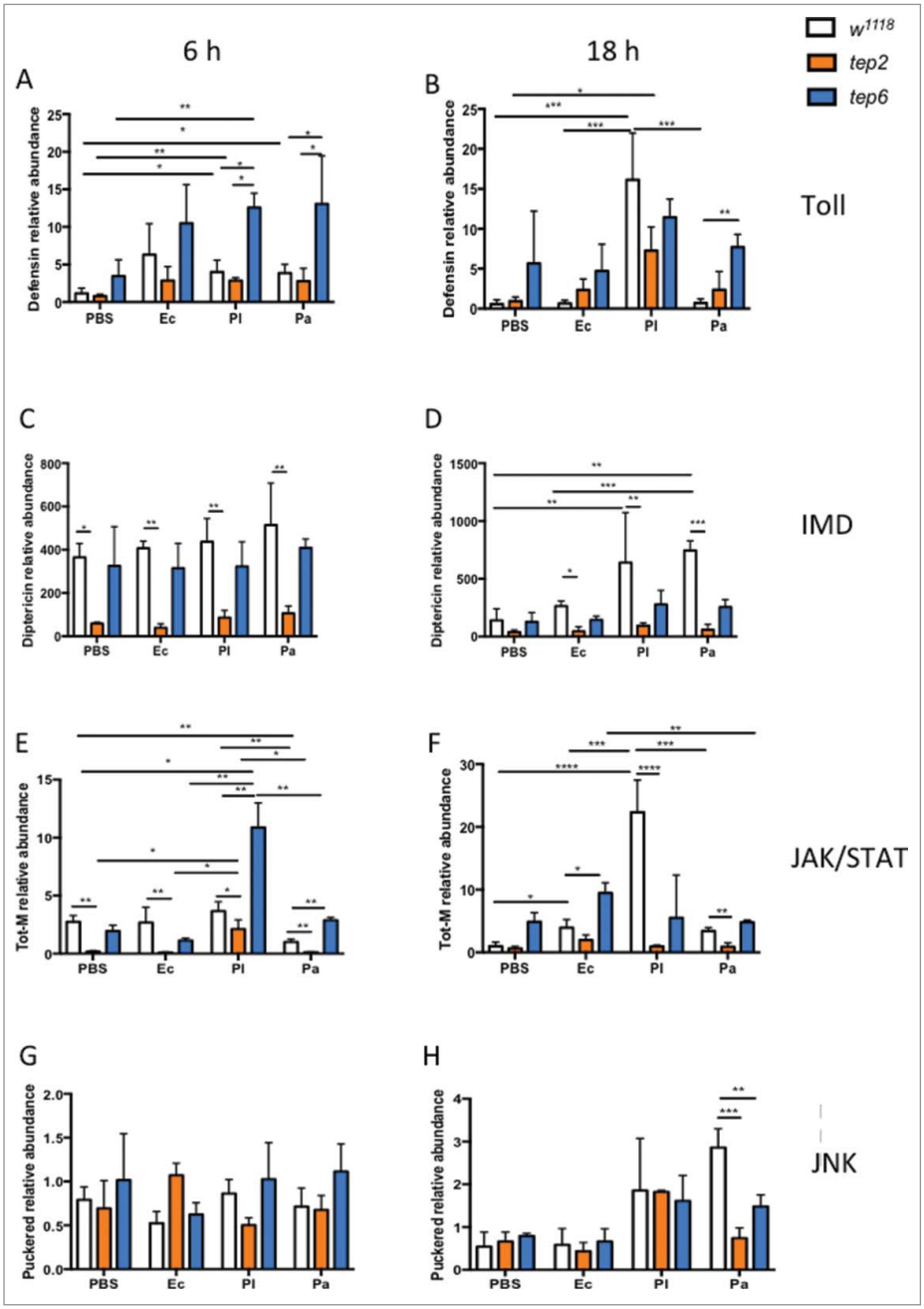

Figure 3. *D. melanogaster Tep2* and *Tep6* differentially regulate the activation of immune pathways against *Photorhabdus*. Transcript levels for (A, B) *Defensin* (Toll pathway), (C, D) *Diptericin* (IMD pathway), (E, F) *Tot-M* (JAK/STAT pathway) and (G, H) *Puckered* (JNK pathway) in loss-of function *tep2* and *tep6* mutants with their corresponding control strain (w^{1118}) at 6 and 18 hpi with 1XPBS, *E. coli* ($_{Ec}$), *P. luminescens* (Pl) or *P. asymbiotica* (Pa) (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 with untreated flies (negative control). Values represent the means from 3 biologic replicates and error bars represent standard deviations. Significant differences are indicated with asterisks; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

the upregulation of *Tep2* and *Tep6* in infected adult flies. For this, we pre-injected the w^{1118} flies with latex beads to ablate the function of hemocytes. A pre-injection with 1X PBS served as control. We found that *Tep2* transcript levels were significantly higher in flies pre-injected with beads or PBS followed by any bacterial treatment at 6 hpi, but only by *Photorhabdus* challenge at 18 hpi (Fig. 4A–B). Moreover, we noticed significant upregulation of *Tep2* in flies pre-injected with PBS compared with flies preinjected with beads followed by infection with *P. luminescens* or *E. coli* (Fig. 4A). There was also significant upregulation of *Tep2* in flies pre-injected with

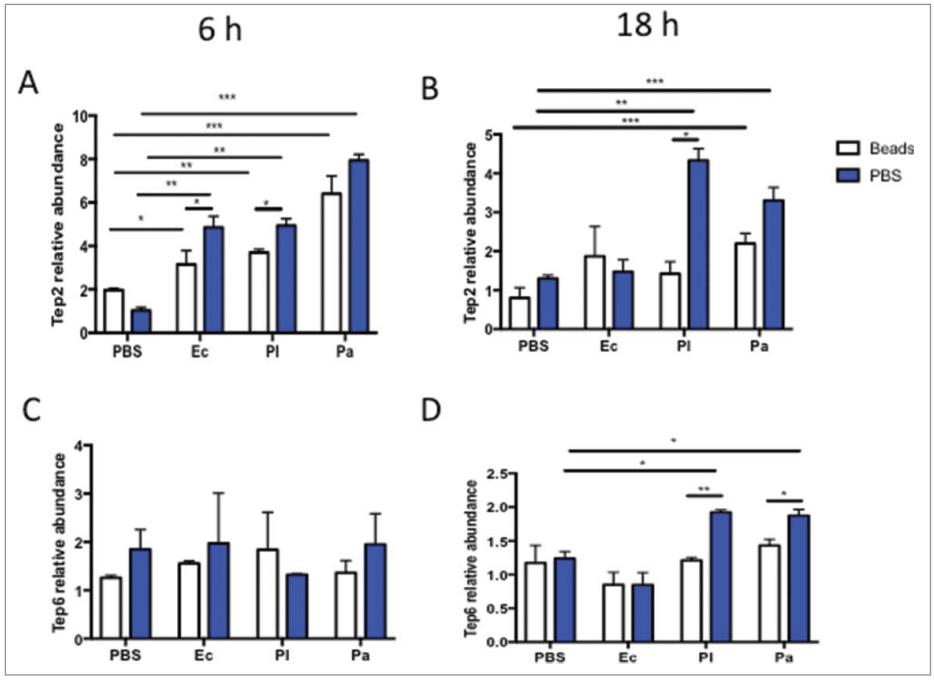

Figure 4. Transcript levels of *Tep2* and *Tep6* are significantly decreased in control flies (w^{1118}) with functionally ablated hemocytes. Transcript levels of (A) *Tep2* and (B) *Tep6* at 6 and 18 hpi with 1XPBS, *E. coli* ($_{Ec}$), *P. luminescens* (Pl) or *P. asymbiotica* (Pa) in w^{1118} flies (n = 5) pre-injected with beads or 1XPBS. Significant differences are indicated with asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Bars show the means from 2 independent experiments and error bars represent standard deviations.

PBS compared with those treated with beads, at 18 hpi with *P. luminescens* (Fig. 4B). Furthermore, we found significant upregulation of *Tep6* in flies pre-injected with PBS than in those injected with beads at 18 hpi with either *Photorhabdus* species (Fig. 4C–D). These results indicate that functional hemocytes are one of the sources of *Tep2* and *Tep6* genes upregulation.

Drosophila Tep2 and *Tep6* mutants have differential number of hemocytes and fewer dead hemocytes against *Photorhabdus* infection

To evaluate whether inactivation of *Tep2* and *Tep6* genes can affect activation of circulating hemocytes, we then investigated the cellular function of *Drosophila* against *Photorhabdus* and *E. coli* bacteria. We first looked at changes in the total number of hemocytes in infected and uninfected flies. Based on the hemocyte counting protocol, the total number of hemocytes was significantly higher in w^{1118} as well as *Tep2* mutants infected with *P. asymbiotica, P. luminescens* or *E. coli* compared with w^{1118} flies injected with PBS (Fig. 5A–B, Fig. S3A). Interestingly, there were significantly more hemocytes in the *Tep2* mutants [(10.52 ± 0.11) $X10^5$ or (3.73 ± 0.46) $X10^4$] than in w^{1118} [(0.44 ± 0.05) $X10^5$ or (1.17 ± 0.34) $X10^4$] flies infected with *P. luminescens* or *E. coli* (Fig. 5A, Fig. S3A). We did not observe any significant change in the number of hemocytes between *Tep6* mutants and control flies infected with *P. luminescens* or *E. coli* bacteria (Fig. 5A, Fig. S3A). Moreover, both *Tep2* [(1.24 ± 0.48) $X10^5$] and *Tep6* mutants [(0.97 ± 0.35) $X10^5$] had significantly fewer hemocytes than the w^{1118} flies [(4.44 ± 1.98) $X10^5$] after *P. asymbiotica* infection (Fig. 5B). However, we observed an increase in hemocyte numbers after *P. asymbiotica* infection in *Tep6* mutants compared with mutants injected with PBS (Fig. 5B). We also evaluated cell viability in *Tep2, Tep6* mutants and w^{1118} flies after infection with *Photorhabdus* and *E. coli.* We observed reduced cell viability in all the strains after *Photorhabdus* and *E. coli* infection (Fig. 5C, Fig. S3B). We found that *tep* mutants contained significantly higher percentage of viable cells compared with w^{1118} flies infected with *Photorhabdus* (Fig. 5C). These data suggest that *Tep2* and *Tep6*, plays an important role in the activation of hemocytes in *Drosophila* flies responding to infection with *Photorhabdus* or *E. coli* bacteria.

Drosophila Tep2 and *Tep6* are required for phagocytosis of *Photorhabdus* or *E. coli* bacteria

To estimate whether inactivation of *Tep2* or *Tep6* affects the phagocytosis of bacteria in *Drosophila*, we injected opsonized inactive *E. coli* bioparticles in *tep* mutants and their control flies. We found that the

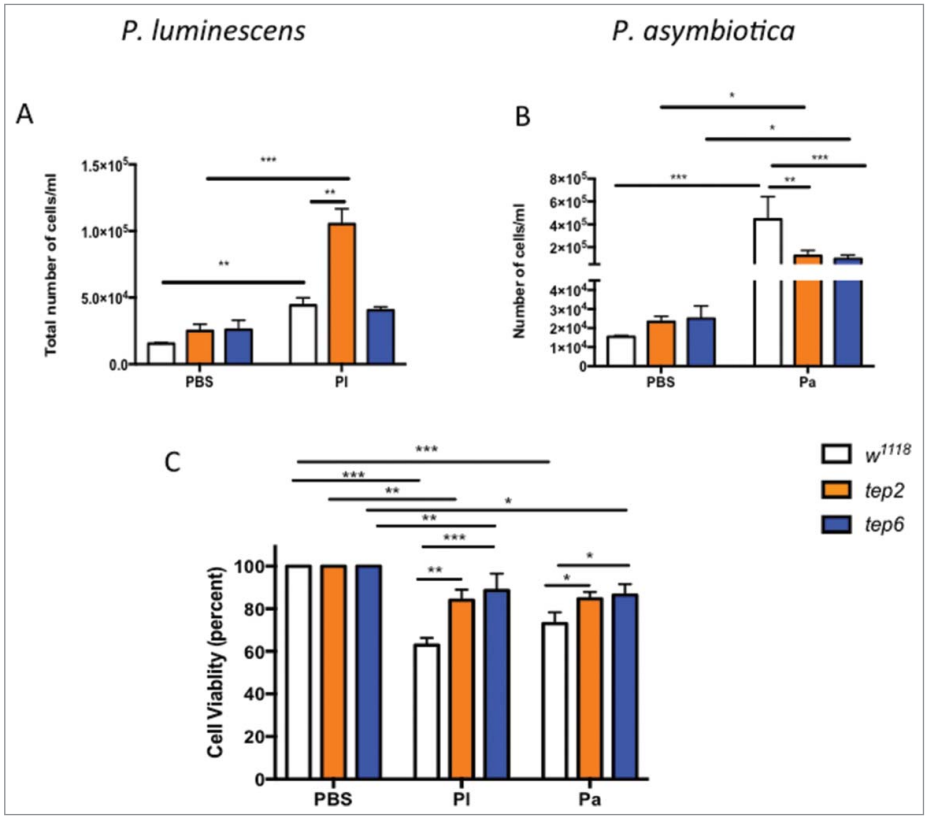

Figure 5. *D. melanogaster tep2* and *tep6* mutants display variable number of total hemocyte counts and increased hemocyte viability compared with control flies (w^{1118}) after *Photorhabdus* infection. According to the hemocyte counting protocol, total number of hemocytes (total cells/ml) in *tep2* and *tep6* mutants with control flies after 18 h of injection with 1XPBS, (A) *P. luminescens* (Pl) or (B) *P. asymbiotica* (Pa). The percentage of total viable cells in the control and *tep* mutant flies at 18 hpi with 1XPBS, (C) *P. luminescens* and (D) *P. asymbiotica*. Significant differences are indicated with asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Bars show the means from 3 independent experiments and error bars represent standard deviations.

phagocytic activity was significantly reduced (~3 times lower) in the *Tep2* and *Tep6* mutants compared with w^{1118} flies at one hpi with *E. coli* (Fig. 6A–B). We also looked at the transcript levels of *Eater* gene, as a marker of phagocytosis,[27] in flies injected with PBS, *E. coli* or *Photorhabdus* bacteria at 6 and 18 hpi. The mRNA levels of *Eater* were significantly higher in control flies infected with *E. coli* at both time points but also with *P. asymbiotica* at 18 hpi (Fig. 6C). We found that *Eater* was significantly upregulated at 6 hpi with *E. coli, P. luminescens* or PBS in w^{1118} flies compared with the *tep* mutants (Fig. 6C). Additionally, we observed that *Eater* mRNA levels were significantly lower in both *tep* mutants compared with control flies at 18 hpi following bacterial or buffer injection (Fig. 6D). In particular, *Eater* mRNA levels were significantly lower in *Tep6* mutants infected with *P. asymbiotica* compared with those injected with PBS at 18 hpi (Fig. 6D). Our data suggest that inactivation of *Tep2* and *Tep6* severely prevents the phagocytic activity in flies against certain bacterial infections.

Tep2 participates in the *drosophila* melanization and phenoloxidase response against *Photorhabdus* infection

To examine whether inactivation of *Tep2* or *Tep6* influence the *in vivo* melanization response in *D. melanogaster*, we visually inspected the wound site at 3 hpi of mutant and control flies with *Photorhabdus, E. coli* or PBS. We observed that w^{1118} flies and *Tep2* mutants exhibited strong melanization response against all injection treatments (Fig. 7A, Fig. S4A). Melanin spots developed in *Tep6* flies following injection with PBS or *E. coli* only (Fig. 7A). We also estimated the phenoloxidase (PO) enzyme activity in the hemolymph plasma of *tep* mutant and control flies injected with the different bacteria. We noticed that the PO activity was significantly reduced in control flies infected with *Photorhabdus* bacteria (Fig. 7B). We found no significant changes in PO activity between *Tep2* or *Tep6* mutant flies and w^{1118} controls injected with PBS or *E. coli* (Fig. 7B, Fig. S4B). Furthermore, *Tep2* mutant flies infected with *P. luminescens* or *P. asymbiotica* had substantially higher

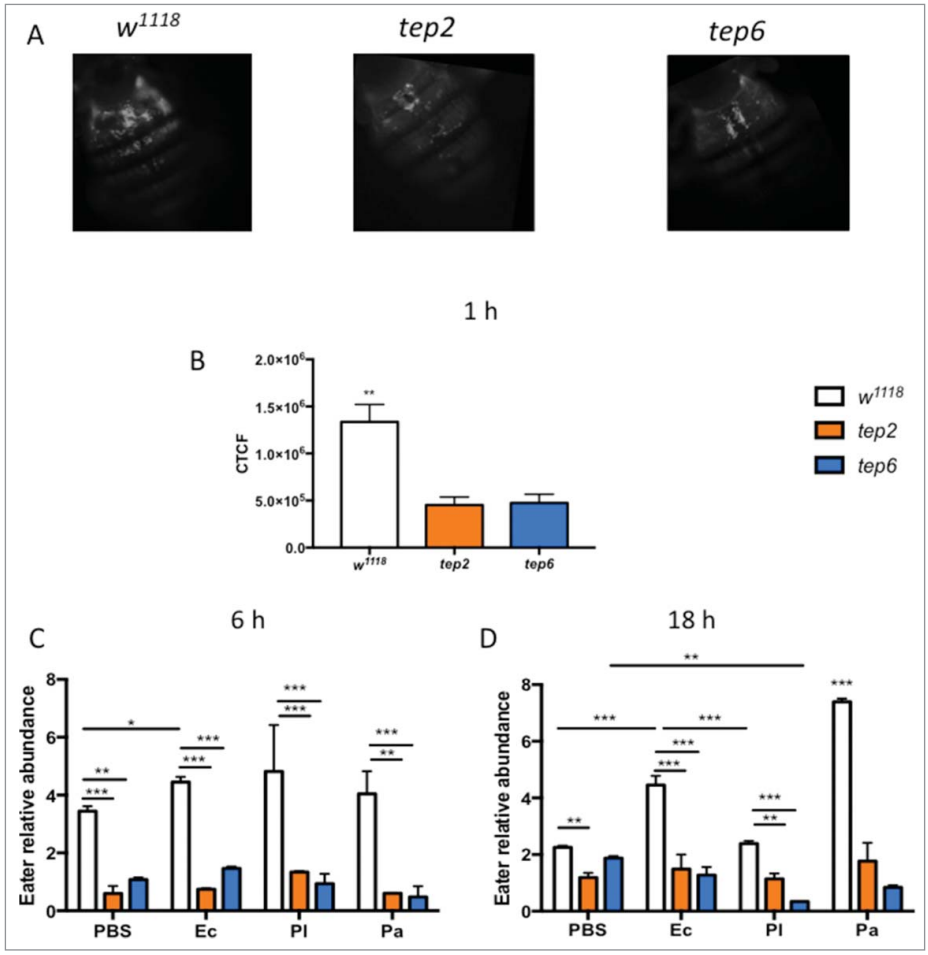

Figure 6. *Tep2* and *Tep6* are essential for the phagocytosis process in *Drosophila*. (A) Representative images of phagocytosis in *tep2* and *tep6* loss-of-function mutants and control flies (w^{1118}) at 1 hpi of lipophilized pHrodo-labeled *E. coli* particles. Images were taken using fluorescence microscopy at 10X magnification. (B) Corrected total cell fluorescence (CTCF) in *tep* mutants and w^{1118} flies (n = 7), 1 h following injection of pHrodo-labeled *E. coli*. Images were processed in ImageJ and CTCF was estimated. Transcript levels of *Eater* in *tep* mutants and w^{1118} flies (n = 5) at (C) 6 h and (D) 18 hpi of 1XPBS, *E. coli* ($_{Ec}$), *P. luminescens* (Pl) or *P. asymbiotica* (Pa). Significant differences are indicated with asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). The means from 2 (*Eater* transcription) -three (Phagocytosis) independent experiments are shown and error bars represent standard deviation.

PO activity than *Tep6* and w^{1118} flies infected with the pathogens. Interestingly, we found that upon *P. asymbiotica* infection, *Tep6* mutants displayed significantly lower levels of PO activity than w^{1118} flies (Fig. 7B). These results suggest that the absence of functional TEP2 in *D. melanogaster* adult flies promotes phenoloxidase activity against infection with pathogenic *Photorhabdus*.

Discussion

Despite remarkable advances in the field of innate immunity, our understanding of the role of TEP molecules in the immune defense of *Drosophila* is mostly unexplored. Recently, we showed the participation of *Tep4* in the humoral and phenoloxidase responses of the fruit fly against *Photorhabdus* infection. Here we investigated the role of other *Tep* genes in the antibacterial immune response of *Drosophila*. Previously, we observed induction of *Tep4* in flies infected with 2 *Photorhabdus* species, therefore we hypothesized that presumably other *Tep* genes might also be activated upon infection with this pathogen.

Previous studies have reported upregulation of *Tep1* and *Tep2* genes but not *Tep6* in adult flies infected with a mixture of *E. coli* and *Micrococcus luteus*.[10,28] Our results are in accordance to the previous studies, as we observe an early upregulation of *Tep2* by *E. coli* bacteria. Transcriptomic analysis has also shown that *Tep1* and *Tep2* are induced following *P. luminescens*, symbiotic *Heterorhabditis* nematodes (carrying *Photorhabdus*) or axenic nematodes (lacking *Photorhabdus*) infection.[8,29] In accordance, upregulation of *Tep1* and *Tep2* in flies after *Photorhabdus*

infection suggests their probable function in the immune response of the fly against the *Photorhabdus* bacteria. Although there are no reports of *Tep3* induction by *Photorhabdus* or its symbiotic nematode partner, recent work has reported that *tep3* loss-of-function mutants are sensitive to *Heterorhabditis* symbiotic nematode infections.[8] No changes in the transcript levels of *Tep3* could be due to its specificity only to nematodes. Also, induction of *Tep6* in response to *P. asymbiotica* indicates a specific function of this molecule against this pathogen. However, as previously reported,[10] we cannot exclude the possibility that the function of TEPs in the fly immune system might be redundant or that TEP molecules might act in combination with other factors to provide efficient levels of protection to the fly against certain pathogens.

Previously TEP molecules have been shown to be expressed in larval plasmatocytes, adult fat body of the head and digestive tract lining at basal levels.[10] Reduced transcript levels of *Tep2* and *Tep6* in flies containing dysfunctional hemocytes upon *Photorhabdus* infection indicates that functional plasmatocytes are one of the major sources for *Tep* gene expression in the adult flies. However, other tissues such as gut or fat body may contribute toward *Tep* gene upregulation when hemocytes are inactive. This could explain the induction of *Tep2* in flies containing non-functional heymocytes in response to *E. coli* or *Photorhabdus* infection.

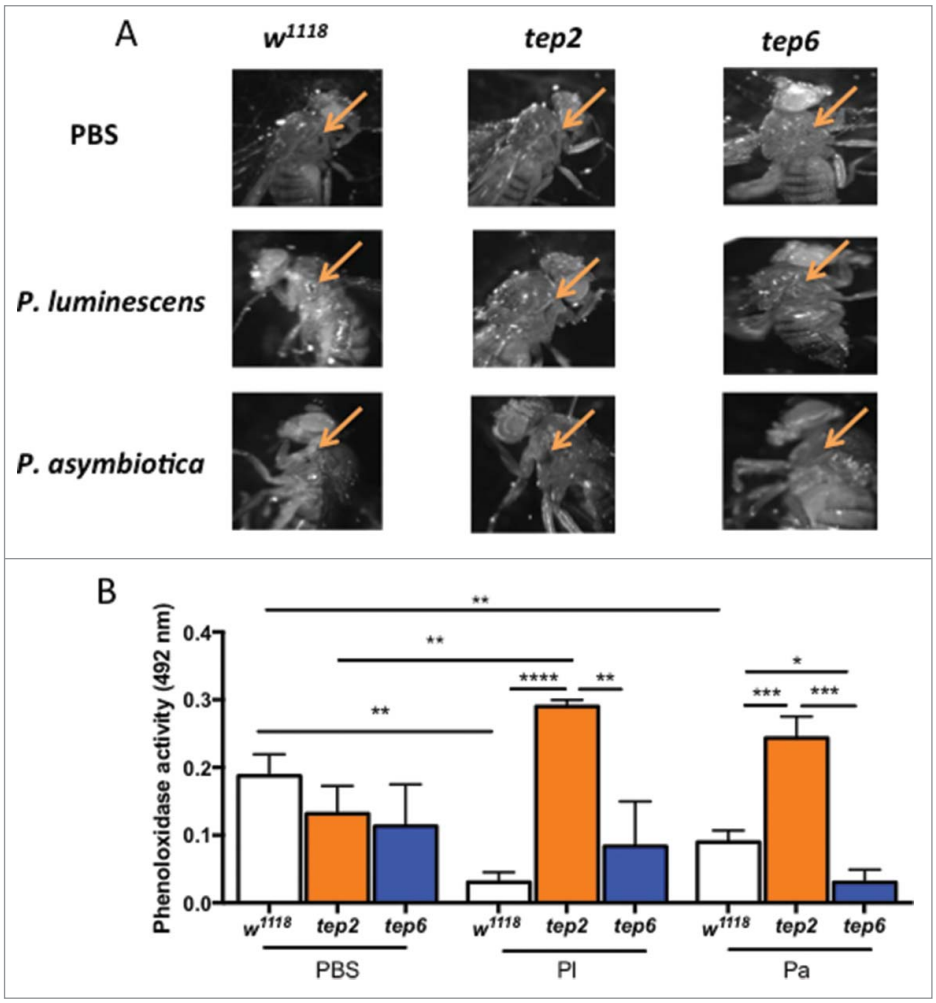

Figure 7. Melanization response and PO activity are elevated in *D. melanogaster tep2* mutants upon *Photorhabdus* infection. (A) Melanization of the wound site in *tep2* and *tep6* loss-of-function mutant flies and their background control strains (w^{1118}) is shown at 10X magnification 3 h after injection with PBS, *P. luminescens* or *P. asymbiotica* bacteria. Arrows indicate the site of injury. (B) PO activity in the hemolymph plasma of *tep2, tep6* mutants and control flies (w^{1118}) at 3 hpi with PBS, *P. luminescens* (PI) or *P. asymbiotica* (Pa) (n = 20 flies) as measured by the optical density at 492 nm after incubation with L-Dopa. Values represent the means from 3 biologic replicates and error bars represent standard deviations. Significant differences are indicated with asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). The means from 3 independent experiments are shown and error bars represent standard deviation.

Inactivation of certain genes in *D. melanogaster* can alter the survival ability of the fly in response to microbial infections.[1] A former study has shown that loss-of-function *Tep2* mutants are susceptible to *Porphyromonas gingivalis* infection.[30] However, another study failed to identify changes in the survival of single, double or triple *tep* mutants in response to Gram-positive and Gram-negative bacterial pathogens as well as to fungal infection.[10] Hence, prolonged survival of *Tep6* mutant flies against *P. luminescens* as well as of *Tep2* and *Tep6* mutants in response to *P. asymbiotica* infection suggests that the survival response is pathogen specific. In addition, the presence of significantly fewer *Photorhabdus* CFU in *Tep2* and *Tep6* mutants could explain their increased survival during the initial and intermediate stages of *Photorhabdus* infection. The finding that *Tep2* mutants are resistant to both *Photorhabdus* and *E. coli* could probably suggest that *Tep2* is evolving in relation to the different pathogen challenges *Drosophila* flies encounter in the wild. Interestingly, it has been previously proposed that *Drosophila Tep2* may have evolved under strong positive selection.[31]

The modulation of immune pathways by *Tep4* [12] and the effect of *Tep4* gene inactivation on the resistance of mutant flies to bacterial infection formed the basis for testing whether TEP2 and TEP6 can also play a central regulatory role in the *D. melanogaster* immune system. No effect on the Toll pathway activation and downregulation of IMD pathway in *Tep2* mutants suggests different mode of actions of *Tep2* and *Tep4* genes. Additionally, our results are in agreement with previous findings as JAK/STAT activation was severely impaired in *Tep2* mutants.[32] Similar to *tep4*, here we find that Toll signaling activity is upregulated in *Tep6* mutants infected with *Photorhabdus*. Stimulation of JAK/STAT in *Tep6* mutants after *Photorhabdus* infection implies that *Tep6* may not participate in the activation of this pathway. Another explanation for the differential induction patterns of the immune signaling pathways in *Tep6* mutants could be the absence of a thioester motif in TEP6 that probably affects its function as an effector molecule. Nevertheless, increased activation of certain pathways in *tep* mutants compared with background controls injected with PBS could suggest that wounding initiates a response in these flies. However, this activation increases in the presence of the non-pathogenic bacteria *E. coli*, as seen in the case of *Tot-M* transcript levels in *Tep6* mutants. In contrast, *Photorhabdus* may interfere with the activation of these pathways by either increasing or suppressing them, as seen with the induction of *Tot-M* in *Tep6* mutants injected with *P. luminescens* or the reduction of *Puc* in *Tep6* mutants infected with *P. asymbiotica*, respectively. Altogether, the 3 TEP molecules-TEP2, TEP4 and TEP6, may modulate immune signaling pathways in discrete ways.

Complement proteins are known to activate mast and basophils in human lungs and blood as an inflammatory and allergic response.[33] Additionally, in *Drosophila*, dramatic change in the number of circulating hemocytes is observed after pathogenic invasion.[34] Nonetheless the function of TEPs in the recruitment and activation of hemocytes in *Drosophila* after bacterial infection is still undefined. The increase in the number of hemocytes in *Tep2* and *Tep6* mutants after *Photorhabdus* or *E. coli* infection indicates that TEP2 and TEP6 are not directly involved in the induction of hemocytes. However, inactivation of either *Tep2* or *Tep6* is not entirely insignificant, as we observed larger numbers of hemocytes in the control flies against *P. asymbiotica* infection. The increase in the number of hemocytes might also be a consequence of other activated TEP molecules, such as TEP4, as we have previously observed a significant decline in the number of hemocytes in the absence of TEP4 following *E. coli* or *Photorhabdus* infection (unpublished).

One of the main evasion strategies of *Photorhabdus* involves targeting and attacking insect hemocytes. *Photorhabdus* can cause morphological changes to hemocytes by affecting the cytoskeletal components, such as actin, that can in turn disturb their normal functions.[35,36] Moreover, *Photorhabdus* pathogens secrete several toxins that can induce apoptosis in the insect hemocytes.[16,37] Increased hemocyte viability in *Tep2* and *Tep6* mutants indicates that inactivation of these *Tep* genes is advantageous for the hemocytes to respond against the *Photorhabdus* insult. This could further support the prolonged survival of the *Tep2* and *Tep6* mutants during the course of *Photorhabdus* infection.

An in vitro study has shown that TEP2 and TEP6 in *D. melanogaster* are involved in the phagocytosis of *E. coli* and *Candida* albicans, respectively.[11] The decreased phagocytosis of inactive *E. coli* particles in the *Tep2* and *Tep6* mutants indicates the significance of TEP2 and TEP6 in the phagocytosis process against *E. coli*. Moreover, the notably reduced transcript levels of *Eater* probably suggests a direct role of TEP2 and TEP6 in this process in response to *Photorhabdus* or *E. coli* infection. We propose that although *Tep2* and *Tep6* mutants contain high numbers of hemocytes after bacterial challenge, due to the inactivation of these 2 *Tep* genes, the phagocytosis function is substantially impaired in the mutants.

We also examined the effect of *Tep2* and *Tep6* on the melanization response, which forms an essential

and rapid cellular immunity process.[38,39] The elevated melanization and phenoloxidase activity in *Tep2* mutants against *Photorhabdus* bacteria points out that TEP2 and TEP4 perform similar immune functions in response to *Photorhabdus* infection in *Drosophila*. This may also account for the reduced number of *Photorhabdus* CFU in the *Tep2* mutants. It could be possible that the growth of *Photorhabdus* bacteria is restricted in the *Tep2* mutants due to increased PO and melanization during the initial phase of infection. In contrast, inactivation of *Tep6* leads to reduced PO and melanization in the flies after *Photorhabdus* infection. The contrasting findings between *Tep2* and *Tep6* mutants may be best explained by the structural difference between the 2 proteins. TEP6, which lacks the thioester motif, regulates phenoloxidase activity and melanization in a different manner than TEP2 and TEP4 molecules, which contain the thioester motif.[10]

In conclusion, we have extended our previous findings that TEPs serve an imperative function in the immune defense of *Drosophila*. The experiments described herein were focused on critical immune responses of fruit fly in response to *Photorhabdus* bacteria. We show that inactivation of *Tep2* and *Tep6* serve a protective and immunomodulatory role against certain insect pathogenic bacteria, such as *Photorhabdus*. Furthermore, our data suggest that different TEP molecules act in a distinct manner in the *Drosophila* antibacterial immune system. It has been shown that disruption of *C5aR* encoding the Complement protein 5a receptor results in increased resistance to acute Gram-negative bacterial infections in mice and this ultimately leads to reduced endotoxic shock.[40] Similarly, we propose that the absence of TEP2 or TEP6 leads to lower levels of inflammation in the host following bacterial infection, which successively modulates their survival ability against potent entomopathogenic bacteria. We anticipate that such studies will lead to a better understanding of the complex mechanism of action of TEP molecules in the antibacterial immune reponse of the fruit fly. These findings could also be applied to insects of agricultural or medical importance.

Materials and methods

Fly and bacterial strains

The following *D. melanogaster* strains were used in the study- w^{1118} (genetic background strain), *Tep2* (f02756, Harvard), and *Tep6* (f03851, Harvard). All strains were kept and amplified for experimentation with instant *Drosophila* media (Carolina Biological Supply) with deionized water. All stocks were maintained at 25°C and a 12:12-hour light:dark photoperiod.

The bacterial strains used were *Photorhabdus luminescens* subsp. laumondii (strain TT01), *P. asymbiotica* subsp asymbiotica (strain ATCC 43949) and *Escherichia coli* (strain K12). Bacteria were cultured in sterile Luria–Bertani (LB) broth for approximately 18–22 h at 30°C on a rotary shaker at 220 rpm. The cultures were then pelleted down, washed and re-suspended in 1x sterile phosphate-buffered saline (PBS, Sigma Aldrich). For infections, bacterial concentrations were brought 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 (NanoDropTM 2000c – Thermo Fisher Scientific).

Infection assays and survival experiment

All procedures were performed as described previously.[12] In brief, 7–10 d old adult flies were anesthetized with CO_2 and then injected in the thorax with 18.4 nl (100–300 CFU) of each bacterial suspension (*P. luminescens, P. asymbiotica* or *E. coli*) or sterile 1XPBS (septic injury control) using a Nanoject II apparatus (Drummond Scientific) equipped with glass capillaries prepared with a micropipette puller (Sutter Instruments). Two replicates of 10 flies each were used for each treatment and survival was recorded at 6-hour intervals and up to 48 hours. Each experiment was replicated at least 3 times.

Bacterial load and gene transcription

All procedures were performed as described previously.[12] Briefly, 4-five adult flies were injected and subsequently frozen at 6 and 18 hours post infection (hpi). DNA was extracted from the frozen flies using DNeasy Blood and Tissue kit (Qiagen) using the manufacturer's protocol. The DNA samples were adjusted to 500 ng for estimating bacterial load. Samples were run in technical duplicates and Quantitative PCRs were performed in twin-tech. semi-skirted 96 well plates on a Mastercycler® ep realplex.[2]Standard curves for each bacterium were used to estimate the bacterial load in infected flies.

For gene transcription studies, total RNA was isolated using the PrepEase RNA spin kit (Affymetrix USB), followed by cDNA synthesis and quantitative RT-PCR (qRT-PCR). ΔΔCt method was used to perform analysis. Data are presented as the ratio between injected flies versus uninfected flies (baseline controls). All the experiments were performed at least 3 times. The list of primers used for the PCR assays are listed in Table 1.

Hemolymph collection, hemocyte counts and viability

Hemolymph was collected from adult female flies (n = 4) at 18 hpi with *P. luminescens, P. asymbiotica, E. coli* or 1X PBS injection; using a modified version of a previously published protocol.[41] Briefly, flies were anesthetized using CO_2 and then injected into the thorax with 2–3 uL of incubation solution [60% Grace's Medium (GM) supplemented with 10% of Fetal Bovine Serum (FBS) and 20% of Anticoagulant Buffer (98 mM NaOH, 186 mM NaCI, 1.7 mM EDTA and 41 mM citric acid, pH 4.5)] using a blunt end needle (16 gauge) fitted with a tubing connected to a 20 ml glass syringe. After 20 minutes of incubation on ice, flies were kept on a petri dish and an incision was made between the 2nd and 3rd abdominal segments. Flies were again injected into the thorax with 5 uL of collection solution (90% of GM supplemented with 10% of FBS). Hemolymph was then collected in a 1.5 mL tube and used for further assays. Hemolymph samples (10 uL) were loaded on a hemocytometer and total numbers of cells as well as the different hemocyte types were estimated using 40X magnification of a compound microscope (Olympus CX21). For cell viability, Trypan blue exclusion assay was performed. All experiments were repeated at least 3 times.

Functional ablation of hemocytes

For ablating the function of hemocytes in *D. melanogaster*, flies were anesthetized with CO_2 and then injected with 69 nL of latex beads (0.3 um diameter, Molecular Probes, Invitrogen) into the thorax using a Nanoject II apparatus (Drummond Scientific) equipped with glass capillaries prepared with a micropipette puller (Sutter Instruments). Latex beads were prepared by washing them with sterile 1XPBS and used 4X concentrated in PBS (corresponding to 5–10% solids). 1XPBS served as control for the first round. After 18 h, the flies were injected again with each bacterial suspension (*P. luminescens, P. asymbiotica* or *E. coli*) or PBS (septic injury control) and used for further assays.

Phagocytosis assay

All procedures were performed as described previously.[22] Briefly, Seven flies from each strain were injected with 50.4 nL of 1 mg/mL pHrodo labeled *E. coli* (Molecular Probes) and allowed to phagocytose at room temperature for 60 min. The flies were fixed ventrally on a glass slide using clear nail paint. Fluorescent images of the dorsal surface were obtained using Nikon ECLIPSE N*i* microscope (10X magnification) fitted with Zyla (ANDOR) 5.5 camera. The images were analyzed using ImageJ software and analyzed. Each experiment was performed 3 times.

Melanization and PO activity

Melanization spots on the site of injury were observed at 3 hpi using a Nikon SMZ18 microscope with Zyla

Table 1. List of primers used in the study.

Gene	Accession number	Primer	Primer Sequence	Tm (°C)
Mcf-1	BX571872	Forward	5′-TTGGCGGGGTGGTAGTCG-3′	61
		Reverse	5′-CAGTTCAGCTTCCTTCTCTAA-3′	
16s rRNA	CP010445	Forward	5′-GGAAGAAGCTTGCTTCTTTGCTGAC-3′	61
		Reverse	5′-AGCCCGGGGATTTCACATCTGACTTA-3′	
*Tcc*C3	FM162591	Forward	5′-CGGCAGCGGAATAAGTCAGAG-3′	61
		Reverse	5′-TCGATGGTCAAGAGGCAAACTG-3′	
RpL32	CG7939	Forward	5′-GATGACCATCCGCCCAGCA-3′	61
		Reverse	5′-CGGACCGACAGCTGCTTGGC-3′	
Tep1	CG18096	Forward	5′-AGTCCCATAAAGGCCGACTGA-3′	61
		Reverse	5′-CACCTGCATCAAAGCCATATTG-3′	
Tep2	CG7052	Forward	5′-TGTTCTGCACCAACAGCGATAC-3′	61
		Reverse	5′-CTGGCGATCCATCAACATTCTT-3′	
Tep3	CG7068	Forward	5′-TCCAAGGGTCCATGTGATGC-3′	61
		Reverse	5′-TAATCCCAACCCGTTCACCG-3′	
Tep6	CG7586	Forward	5′-CGCCTTCCTGAACGAAACAA-3′	61
		Reverse	5′-GAGGCTTATCGGTCTGCACAA-3′	
Defensin	CG1385	Forward	5′-CGCATAGAAGCGAGCCACATG-3′	56
		Reverse	5′-GCAGTAGCCGCCTTTGAACC-3′	
Diptericin	CG10794	Forward	5′-ACCGCAGTACCCACTCAATC-3′	57
		Reverse	5′-CCCAAGTGCTGTCCATATCC-3′	
Turandot-M	CG14027	Forward	5′-GGTTTGCTTCAGCGTTCCAAAAAGTCATAACC-3′	61
		Reverse	5′-ATTAAAACAATATTAACCAGTGAATAATTGAG-3′	
Puckered	CG7850	Forward	5′-GGCCTACAAGCTGGTGAAAG-3′	61
		Reverse	5′-AGTTCAGATTGGGCGAGATG-3′	
Eater	CG1624	Forward	5′-ATAACGATCCATCTAACCGATGTGT-3′	57
		Reverse	5′-GATTGGCAGGTTCCTCGACTAC-3′	

(ANDOR) 5.5 camera. Images were analyzed using Nikon Software Suite at 10X magnification. PO activity was measured as described previously (Shokal and Eleftherianos, 2016). Briefly, at 3 hpi the injected flies (n = 20) were placed on a spin column (Pierce, Thermo fisher) containing 2.5X protease inhibitor (Sigma) and covered with 5 4 mm glass beads (VWR). They were centrifuged at 4°C and 13,000 rpm for 20 min. Protein concentrations were then adjusted using a BCA test. A mixture of 15 μg of protein (diluted in 2.5x protease inhibitor) with 5 mM $Cacl_2$ was added to L-DOPA solution (15 mM in phosphate buffer, pH 6.6) making a final volume of 200 μL. The absorbance (OD 492 nm) for each sample was measured after 36 min incubation at 29°C in the dark against a blank control. Each experiment was performed in biologic duplicates and repeated 3 times.

Statistical analysis

All statistics were performed using the GraphPad Prism7 software. Analysis of survival experiments was conducted using a Log-rank (Mantel-Cox) and Chi-square tests. Unpaired 2-tailed t-test and 2-way analysis of variance (ANOVA) with a Tukey post-hoc test for multiple comparisons were used for analyzing bacterial load, gene expression data, hemocyte cell counts, cell viability and PO activity results. p values below 0.05 were considered statistically significant.

Abbreviations

Hpi	hours post infection
PO	Phenoloxidase
TEPs	Thioester-containing proteins

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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