

# Analysis of Thioester-Containing Proteins during the Innate Immune Response of *Drosophila melanogaster*

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## Key Words

*Drosophila melanogaster* · Gene disruption · Genome evolution · Genomic sequence analysis · In situ hybridization · Innate immunity · Insects · Invertebrates · Thioester-containing proteins

## Abstract

Thioester-containing proteins (TEPs) are conserved proteins among insects that are thought to be involved in innate immunity. In *Drosophila*, the *Tep* family is composed of 6 genes named *Tep1–Tep6*. In this study, we investigated the phylogeny, expression pattern and roles of these genes in the host defense of *Drosophila*. Protostomian *Tep* genes are clustered in 3 distinct branches, 1 of which is specific to mosquitoes. Most *D. melanogaster* *Tep* genes are expressed in hemocytes, can be induced in the fat body, and are expressed in specific regions of the hypodermis. This expression pattern is consistent with a role in innate immunity. However, we find that *TEP1*, *TEP2*, and *TEP4* are not strictly required in the body cavity to fight several bacterial and fungal infections. One possibility is that *Drosophila* TEPs act redundantly or that their absence can be compensated by other components of the immune response. TEPs may thus provide a subtle selective advantage during evolution. Alternatively, they may be required in host defense against specific as yet unidentified natural pathogens of *Drosophila*.

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## Introduction

The identification of pathogenic threats, either by pattern recognition receptors or by sensing of microbial virulence factors, is crucial to innate immune function, which only relies on germ-line-encoded receptors [1]. In vertebrates, complement factors directly bind to the surface of pathogens thereby initiating their destruction [2]. In the alternative activation pathway of vertebrate complement, surfaces are bound nonspecifically by complement proteins. The subsequent activation of complement is prevented by specific inhibitors expressed on self surfaces such as CD46 and CD55, which inhibit the formation of the C3 convertase responsible for the activation of the C3 complement factor [3]. These inhibitors are missing on nonself surfaces and complement activation ensues. Thus, the alternative complement pathway has been suggested to recognize missing self [3]. Complement proteins belong to the thioester-containing protein (TEP) family, which is present in a wide variety of species, ranging from deuterostomes to protostomes, suggesting that the complement system has evolved and adapted over a period of up to 1 billion years [4]. Phylogenetically, TEPs from insects share sequence similarities with both the vertebrate complement factors C3/C4/C5 and the  $\alpha_2$ -macroglobulin family of protease inhibitors. These TEPs contain a central hypervariable region, which corre-

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sponds to the bait domain of  $\alpha_2$ -macroglobulin and to the anaphylatoxin domain in C3. Most TEPs share the common 4-amino-acid sequence (CGEQ) defining the thioester site, which allows the formation of a covalent bond to microbial surfaces.

*Drosophila melanogaster* is a valuable model to decipher the mechanisms of insect innate immunity [5, 6]. The TEP family was first investigated at a time when pattern recognition receptors able to trigger the systemic immune response had not yet been identified. Its relative similarity to the complement-like system described in invertebrates such as the sea urchin, tunicates and arthropods made them good candidates for missing self receptors [7]. The *Tep* family in *Drosophila* is composed of six genes (*Tep1–Tep6*), one of which, *Tep5*, does not appear to be expressed [7]. TEP6 (also known as macroglobulin-complement related, MCR) is an exception in this *Drosophila* family because the thioester binding site is likely to be nonfunctional as the cysteine residue of the motif has been replaced by serine.

TEPs have been mostly studied in the mosquito *Anopheles gambiae* where TEP1 (aTEP1) was shown to act as a bona fide opsonin to promote phagocytosis of Gram-positive and -negative bacteria [8]. aTEP1 is structurally related to the C3 complement factor, is expressed in hemocytes, and secreted in the hemolymph, where it can bind to the surface of bacteria [8, 9]. Other studies have highlighted the role of aTEP1 in the defense against *Plasmodium* infections [9]. aTEP1 binds to the surface of ookinetes and promotes their lysis and melanization. In *D. melanogaster*, *Tep* genes are expressed at basal levels and are upregulated upon immune challenge [7, 10, 11]. The only functional data available so far revealed that TEP2, TEP3, and TEP6 bound to *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*, respectively, thereby promoting their phagocytosis by cultured S2 cells [12]. Unexpectedly, the binding of TEP6 to *C. albicans* indicated that a functional thioester motif is not essential to interact with microbial surfaces [12].

Here the function of the *Drosophila Tep* family was investigated using several approaches. First, a phylogenetic analysis of protostomian *Tep* members revealed the existence of three major subgroups, one of which appears to be specific to mosquitoes. Second, we examined the expression pattern of *Tep* genes. We found that *Tep* genes are essentially expressed in hemocytes, in some barrier epithelia, and in the fat body. Finally, we tested mutants affecting one or several *Tep* genes in various infection models and failed to detect a specific susceptibility phenotype. Taken together, our results are compatible with a

role for TEPs in innate immunity, even though functional redundancy and/or the lack of a relevant infection model may have prevented us from identifying their exact role in vivo.

## Materials and Methods

### Bioinformatics and Phylogenetic Analysis

Sequences of the TEPs or TEP homologues presented have been retrieved from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>), Ensembl ([www.ensembl.org](http://www.ensembl.org)), FlyBase (<http://flybase.org/blast/>), and JGI (<http://genome.jgi-psf.org/>) data bases using the sequence retrieval system or/and BLAST (basic local alignment search tool) [13]. Alignments were carried out using clustal W [14], MUSCLE 3.7 [15; [www.phylogeny.fr](http://www.phylogeny.fr)] or COBALT (constraint-based multiple alignment tool; [www.ncbi.nlm.nih.gov/tools/cobalt](http://www.ncbi.nlm.nih.gov/tools/cobalt)). The identification numbers and the detailed species are presented in online supplementary tables S1 and S2 ([www.karger.com/doi/10.1159/000321554](http://www.karger.com/doi/10.1159/000321554)).

Phylogenetic trees were constructed on the basis of amino acid differences using PhyML 3.0, applying the approximate likelihood ratio test and bootstrapping procedure with a minimum of 100 bootstraps [15; [http://www.phylogeny.fr/version2\\_cgi/phylogeny.cgi](http://www.phylogeny.fr/version2_cgi/phylogeny.cgi)], fast minimum evolution, neighbor joining, and cobalt tree [16; [www.ncbi.nlm.nih.gov/blast/treewiew](http://www.ncbi.nlm.nih.gov/blast/treewiew)]. Reliability of the trees was assessed by bootstrapping and comparison between the methods. The median bootstrap values for the phylogenetic trees were not <98%.

### Fly Strains

Flies were raised on standard cornmeal agar at 25°C. *w<sup>-</sup>* A5001 flies were used as wild-type controls for most of the experiments [17]. *Tep2* (d11521) and *Tep3* (d03976) have been generated in an A5001 background [17]. The *Tep4* mutant belongs to another P-insertion mutant library (EY04656) [18]. The *Tep2,3* double mutant was generated by deleting the region between *Tep2* and *Tep3* by recombining the FRT sites contained in the P-transposable elements (Flp flippase) inserted in the respective genes. We checked the deletion by PCR using appropriate sets of primers. The *Tep2,3,4* triple mutant was obtained by recombination of the *Tep2,3* double mutant with the *Tep4* mutant. UAS-*Tep1* RNAi (ML2D) transgenic flies were generated by Dr. Marie Lagueux. Further fly strains include *key<sup>c02831</sup>* mutants (A5001 background) [Ferrandon, unpubl. data], *Dif<sup>1</sup>* mutants [19], and *spz<sup>rm7</sup>*. The *hsp-Gal4* driver was used to overexpress ubiquitously the different transgenes under heat shock conditions. *Tep1*-GFP are transgenic flies in which the promoter of the *Tep1* gene is fused to GFP-coding sequences.

### Microbial Strains

The following microbes were used: *E. coli* (1106), *Micrococcus luteus* (CIP A270), *Enterobacter cloacae*, *S. aureus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Mycobacterium marinum* (a kind gift from Dr. Herman Spaink), *Streptococcus pyogenes*, and *Beauveria bassiana* (80.2 strain). Infections were performed at 25°C with the exception of *M. marinum* and *B. bassiana*, which were done at 29°C.

### Quantitative RT-PCR

This analysis was done as previously described [20]. Each experiment was done at least three times. For quantitative PCR, we used the following primers shown from 5' (left) to 3' (right): (i) *Tep1*: forward: AGTCCCATAAAGGCCGACTGA, reverse: CACCTGCATCAAAGCCATATTG; (ii) *Tep2*: forward: TGTTCTGCACCAACAGCGATAC, reverse: CTGGCGATCCATCAACATTCTT; (iii) *Tep4*: forward: GCTGCAGAACCAGATCGAAATC, reverse: ATGACTTTGGCGACGTCTTGAT, and (iv) *Tep6*: forward: CGCCTTCCTGAACGAAACAA, reverse: GAGGCTTATCGGTCTGCACAA. The following plasmids containing full-length cDNA (generated by Dr. Marie Lagueux) served as standards: *Tep1*: pML115 in pUAS<sub>t</sub>, *Tep2*: pML91 in pOT2, and *Tep4*: pML208 in PGEM easy. *Tep6*: LD23292 in pOT2 was purchased from the Berkeley Drosophila Genome Project. Quantitative PCR data obtained using these primer couples were then normalized against the values obtained by measuring the expression of the gene encoding ribosomal protein 49.

### In situ Hybridization

In situ hybridization (ISH) on larval whole-mount tissues and on circulating hemocytes has been reported previously [7, 10, 11]. In situ RNA hybridization on sections was carried out as described [21]. The same plasmids described for quantitative RT-PCR were also used for ISH. We generated sense and antisense probes for *Tep2*, *Tep4*, and *Tep6*. Experiments shown are representative of at least two independent experiments.

### Septic Injury and Survival Experiments

Survival experiments were performed as previously detailed [20]. Batches of 20 wild-type and mutant flies were challenged by septic injury using a needle previously dipped in a concentrated solution of an overnight bacterial culture. For some bacterial infections, we injected specific dilutions for *S. aureus* (OD<sub>600</sub> = 0.1), *S. pyogenes* (≈OD<sub>600</sub> = 17), and *M. marinum* (500 CFU). Survival experiments were performed with the fungus *B. bassiana* as previously described [22]. The vials containing the challenged flies were then transferred to an incubator and kept at 25 or 29°C (according to the microbial strain) and surviving flies were counted every few hours. Experiments shown are representative of at least three independent experiments (for *M. marinum* infections, refer to [23]). Survival data have been analyzed using the log-rank test provided in the Prism<sup>®</sup> software.

### Phagocytosis Assay

Batches of 10 wild-type (A5001) or *Tep2,3,4* mutant flies were injected with either PBSx1 or latex beads; 16 h later, flies were injected with pHrodo™ *E. coli* BioParticles<sup>®</sup> conjugate for phagocytosis (Invitrogen), prepared according to the manufacturer's instructions. Thirty minutes after injection, flies were dissected, carcasses were rinsed with PBS, mounted in Vectashield and observed with a fluorescent microscope (Axio Scope; Zeiss).

Experiments shown are representative of two independent experiments. For each experiment, several flies were scored independently by two persons in a 'blind' manner.

## Results

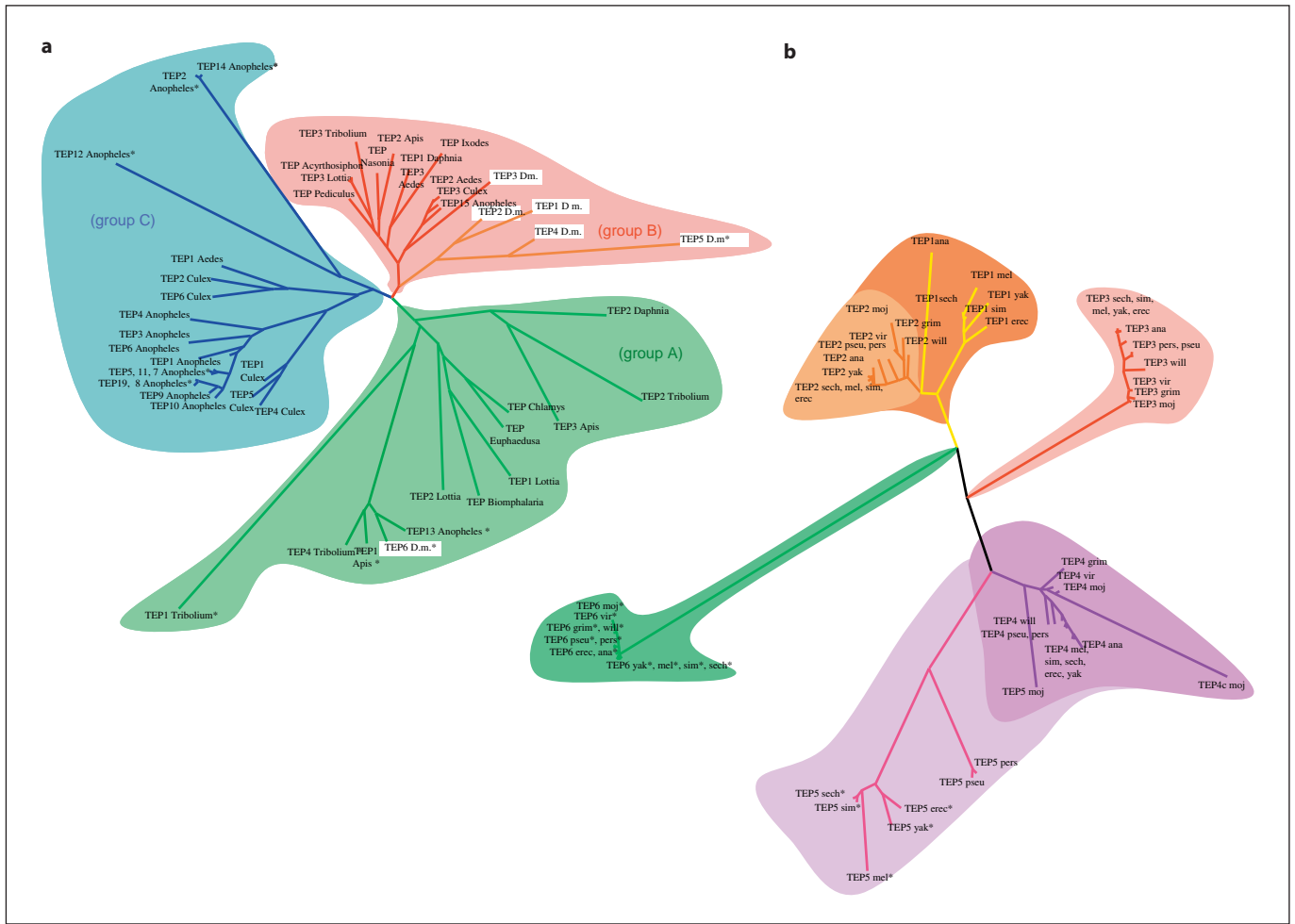
### Phylogenesis of Tep Genes among Protostomes

We have analyzed the phylogeny of *Teps* among various ecdysozoa (insects, chelicerates, and crustaceans), and lophotrochozoa (gastropods and bivalves) for which genome data are available (online suppl. table S1; online suppl. fig. S1). Three main groups emerged from the phylogenetic analysis (fig. 1a). Group A includes the *D. melanogaster Tep6* and *A. gambiae Tep13* (fig. 1a). It contains *Tep* genes from both ecdysozoa and lophotrochozoa, suggesting that this pan-protostomian *Tep* group has emerged first during evolution. Indeed, the only *Tep* genes retrieved from the gastropod sequences present in our panel, i.e. *Biomphalaria glabrata*, *Euphaedusa tau*, and *Chlamys farreri*, belong to this group. Note also that all *Tep* genes from *Tribolium castaneum* and *Apis mellifera*, except *Tribolium-Tep3* and *Apis-Tep2*, belong to group A. In contrast, no *Tep6* gene sequence was found in the chelicerate *Ixodes scapularis*, possibly because its recently sequenced genome might still have been incomplete. Alternatively, this branch of arthropods may have lost this gene family, as may be the case for *Aedes aegypti*, *Culex pipiens*, *Nasonia vitripennis*, *Acyrtosiphon pisum*, and *Pediculus humanus corporis*. The absence of a functional thioester motif in DmTEP6, Anopheles TEP13, Apis TEP1, and Tribolium TEP4 is not a defining feature of group A, as other members of the group, including Tribolium *Tep2* and Apis *Tep3*, encode a functional motif.

Group B can be divided into two branches, one related to *D. melanogaster Tep3*, and one restricted only to *D. melanogaster Tep1*, *Tep2*, *Tep4*, and *Tep5* (fig. 1a). The *DmTep3*-related branch clusters species from both ecdysozoa and one lophotrochozoa, but in contrast to group A, these sequences have been well conserved during evolution. This suggests that the *Tep* genes belonging to this branch share a common ancestral function that may be under stronger selective pressure in these various organisms.

Most strikingly, group C is solely composed of *Tep* genes from mosquito species namely, *A. gambiae*, *A. aegypti*, and *C. pipiens*. Importantly, *Anopheles Tep1*, which plays an essential role in the mosquito host defense against parasites [9], belongs to this cluster of mosquito-related *Tep* genes. Of note, many TEP members from *A. gambiae* (TEP2, 5, 7, 8, 11, 12, 14, and 19) also contain a potentially nonfunctional thioester site, as reported for some TEPs in the two other groups.

The second branch of group B contains only *D. melanogaster Tep* genes (*Tep1*, *Tep2*, *Tep4*, and *Tep5*) and there-



**Fig. 1.** Phylogenetic analysis of *Tep* genes. **a** Phylogenetic analysis of available protostomian *Tep* sequences. **b** Phylogenetic analysis of *Tep* sequences issued from the 12 sequenced *Drosophila* species. The references for all sequences used to generate the phylogenetic

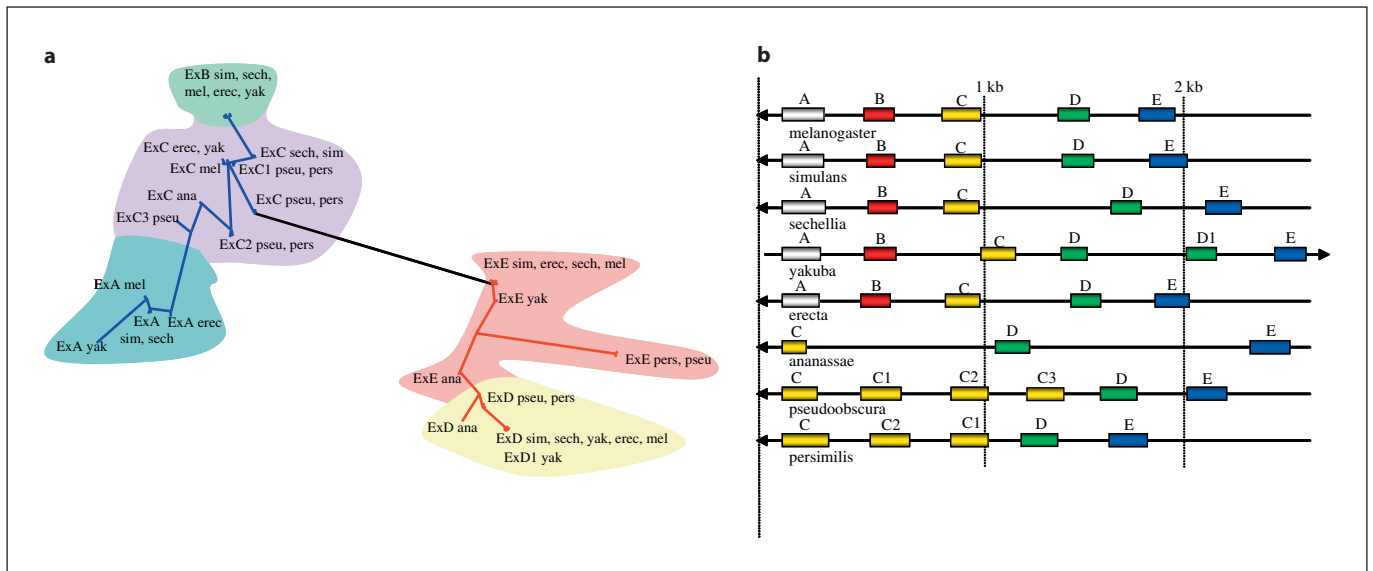
trees are provided in online suppl. table S1 (see also online suppl. fig. S1 for a phylogenetic tree of the species used for the interpretation of the *Tep* phylogenetic trees). Sequences indicated by an asterisk encode a nonfunctional thioester motif.

fore seems restricted to Brachycera, and possibly only to the Drosophilidae (fig. 1b; online suppl. fig. S1). This suggests that these *Tep* genes appeared more recently during evolution and thus may reflect a specific adaptation of Brachycera/Drosophilidae. Additionally, these genes are phylogenetically clustered together, suggesting that they emerged recently, most likely from a duplication of the *DmTep3* ancestor.

A more detailed analysis of the phylogeny of the Drosophilidae branch is possible thanks to the sequencing of 12 *Drosophila* species (online suppl. fig. S1) [24]. *Tep6* genes, followed to a lesser extent by *Tep3* genes, display a high level of sequence similarity (fig. 1b). Taking into account the phylogeny described above (fig. 1a: groups A

and B), this indicates that these two genes may encode the ancestral functions of *Tep* genes. It is likely that a gene duplication event generated both the current Drosophilidae *Tep3* gene and a precursor that underwent further duplications to generate *Tep1* and *Tep2* on the one hand, and *Tep4* and *Tep5* on the other hand. The duplication event that generated *Tep1* occurred just before the diversification of the melanogaster group as *Tep1* is lacking in other Drosophilidae.

*Tep5* (CG13079) likely emerged from a duplication of *Tep4* before the separation of the obscura and melanogaster groups (fig. 1b; online suppl. fig. S1), as this gene is absent in the willistoni group of Sophophora (which includes *D. melanogaster*) and in the other branch of Dro-



**Fig. 2.** Phylogenesis of exon 5 of *Tep2*. **a** Phylogenetic analysis of the exons 5 of *Drosophila Tep2s*. **b** Positions of the exons 5 on the genome of *Drosophila* species. The letters A, B and C refer to those given in FlyBase for *melanogaster* species; we have inverted D and E to follow the disposition on the chromosome.

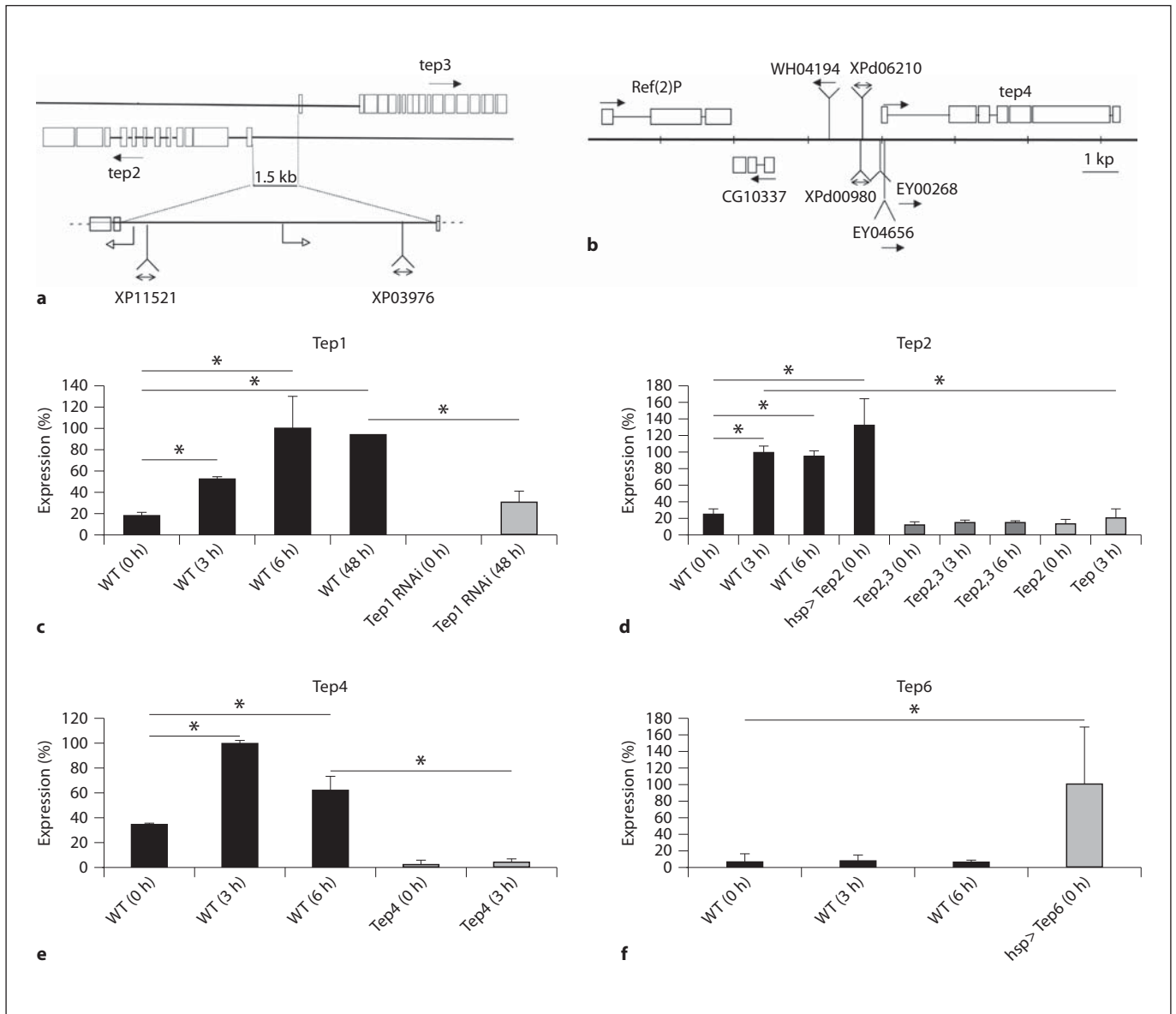
sophilidae, namely *Drosophila*. Interestingly, this gene does not encode an active thioester site in the melanogaster group, while no mutation of this site occurred in the other *Drosophila* species. This suggests that the loss of this thioester site occurred secondarily in the melanogaster group. Interestingly, there is no evidence that the *D. melanogaster Tep5* gene is expressed as no hints of its expression could be found in FlyBase where large-scale genomic expression data, including RNAseq data, are available (<http://flybase.org/>). We cannot however exclude that this gene may be expressed only in a few cells or under very specific conditions, such as the encounter with a specific pathogen. Thus, it will be interesting to determine whether the expression of other *Drosophila* species *Tep5* genes is more readily detectable. It will also be worthy to examine, when released, the genome of other Brachycera, such as *Musca domestica* or *Glossina morsitans*, to determine whether the *Tep1,2,4* branch of group B is specific to Drosophilidae or Brachycera.

The *Tep2* gene is transcribed into five isoforms (A–E) by alternative splicing of exon 5, which corresponds to the variable region of TEP2 [7], that is the region orthologous to the bait domain of the  $\alpha_2$ -macroglobulins or the anaphylatoxin of C3 complement factor (C3a). These five isoforms are found only in the Sophophora [melanogaster (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. ananassae*, and *D. erecta*)] and obscura (*D. pseudoobscura*

and *D. persimilis*) groups (fig. 2). The phylogeny of exon 5 within these species suggests that exons C and E might have arisen from a single duplication from an ancestral exon (fig. 2a). Exon E underwent an early duplication event before the separation of the melanogaster and obscura groups, thus yielding exon D. In contrast, exon C appears to have undergone independent duplication events in the obscura and melanogaster subgroups. Interestingly, exon C did not undergo such an event in *D. ananassae*, a member of the melanogaster group but not of the melanogaster subgroup (online suppl. fig. S1), unless duplicated genes were subsequently lost (fig. 2b). In the melanogaster subgroup, exon C yielded independently (fig. 2a) two further exons A and B. It is worth underscoring the possible adaptive value of such an evolution of exon C that generated multiple additional exons during the differentiation of the Drosophilidae.

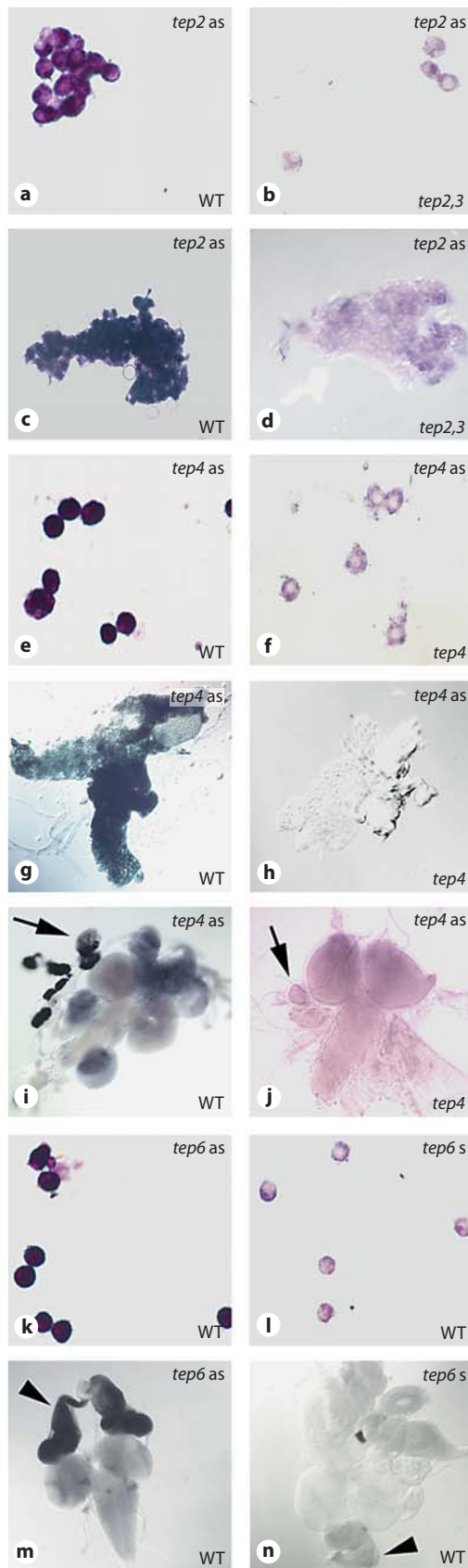
#### *The Induction of Tep Gene Expression Is Blocked in the Corresponding Mutants*

To investigate the function of TEPs in innate immunity of *Drosophila*, we took advantage of loss of function fly lines described below. *Tep2* and *Tep3* are 1.5 kb apart from each other in an opposite orientation and transposable elements are inserted next to each transcription start site (fig. 3a). Transposable elements are also inserted in the *Tep4* genomic region (fig. 3b). We also used an RNAi



**Fig. 3.** Modulation of the expression of *Tep* genes in adults after septic injury. **a, b** *Drosophila* *Teps* are located on the left arm of the second chromosome. **a** *Tep2* (CG7052) and *Tep3* (CG7068) map to cytogenetic position 28C1. *Tep2* and *Tep3* are 1.5 kb apart. The modified XP transposable element XP11521 is inserted 22 bp upstream of the transcription-starting site of *Tep2*, whereas the XP03976 element is inserted 121 bp downstream of the transcription-starting site of *Tep3*. We have generated a double mutant for *Tep2* and *Tep3*, *Tep2,3*, by FLP-mediated recombination between the FRT elements carried on the XP elements. **b** *Tep4* (CG10363) maps to cytogenetic position 37F1. Transposable elements inserted in this region are shown. We have used EY04656 as a mutant for *Tep4* as this modified P element is inserted in the initiation codon of the *Tep4* gene. In the following experiments, we used an

RNAi transgenic line to knock down *Tep1* (CG18096). **c-f** Steady-state transcript levels of *D. melanogaster* *Tep* genes were measured by quantitative RT-PCR before and after infection with a mix of *E. coli* and *M. luteus*. These experiments are representative of at least two independent experiments. 0 h = Non-infected flies; 3 h/6 h/48 h = flies 3/6/48 h after infection; WT = wild type. Flies were frozen for experiments 3, 6, and 48 h after infection. Gene expression was normalized against *rp49* gene expression and the results are expressed as percentage of maximal expression: 6 (*Tep1*; **c**) and 3 h after infection (*Tep2* and *Tep4*; **d, e**). Expression of a *Tep6* transgene under the control of a heat shock promoter using the UAS-GAL4 system (*hsp>Tep6*) after heat shock (**f**). \*  $p < 0.05$ . **c** The induction of *Tep1* is decreased by the expression of an RNAi transgene targeting specifically this gene.



fly line to interfere with *Tep1* transcription. Previous transcriptomic studies [7, 10, 11] have shown that *Tep2* and *Tep4* genes are highly upregulated in adults upon bacterial challenge. In addition, we also report here an increased expression of the *Tep1* and *Tep3* genes (fig. 3c; online suppl. fig. S2). Moreover, we found that only *Tep6* expression did not increase after septic injury with a mixture of *E. coli* and *M. luteus* (fig. 3f). The increased expression of *Tep1*, *Tep2*, *Tep3*, and *Tep4* genes after an immune challenge was impaired in *Tep2* and *Tep4* loss-of-function fly lines (fig. 3d, e; online suppl. fig. S2), as well as in *Tep1* RNAi flies where the expression of *Tep1* was reduced by 70% (fig. 3c). The *Tep6* loss-of-function allele is larval lethal and therefore we did not attempt any further investigation in this study.

#### Expression of Tep Genes in Potential Immune Tissues of *D. melanogaster* Adult Flies and Larvae

To identify the tissues in which *Tep* genes are expressed before or after an immune challenge, we analyzed the expression pattern of several *Tep* genes in adult and larval tissues. The expression pattern of *Tep2*, *Tep3*, *Tep4*, and *Tep6* was assessed by whole-mount ISH of larvae (fig. 4; online suppl. fig. S2) and by ISH of histological sections in adult flies (fig. 5). To investigate the expression pattern of *Tep1*, we took advantage of a transgenic fly line expressing the GFP reporter under the control of the *Tep1* promoter. We found that *Tep1*-GFP and *Tep4* transcripts are expressed in the lymph glands (the larval hematopoietic organ; fig. 4i, j, and data not shown). *Tep1*-GFP was also detected in the proventriculus, the larval plasmatocytes, and the larval fat body (online suppl. fig. S3). We also found that *Tep2*, *Tep3*, *Tep4*, and *Tep6* are expressed in larval plasmatocytes (fig. 4a, b, e, f, k, l; online suppl. fig. S2). Additionally, *Tep1*-GFP, *Tep2*, and *Tep4* are expressed at basal levels in the fat body, and this expression is increased after a septic injury (*E. coli* + *M. luteus*; fig. 4c, d, g, h; online suppl. fig. S3, and data not shown). Since the fat body and plasmatocytes are responsible for the

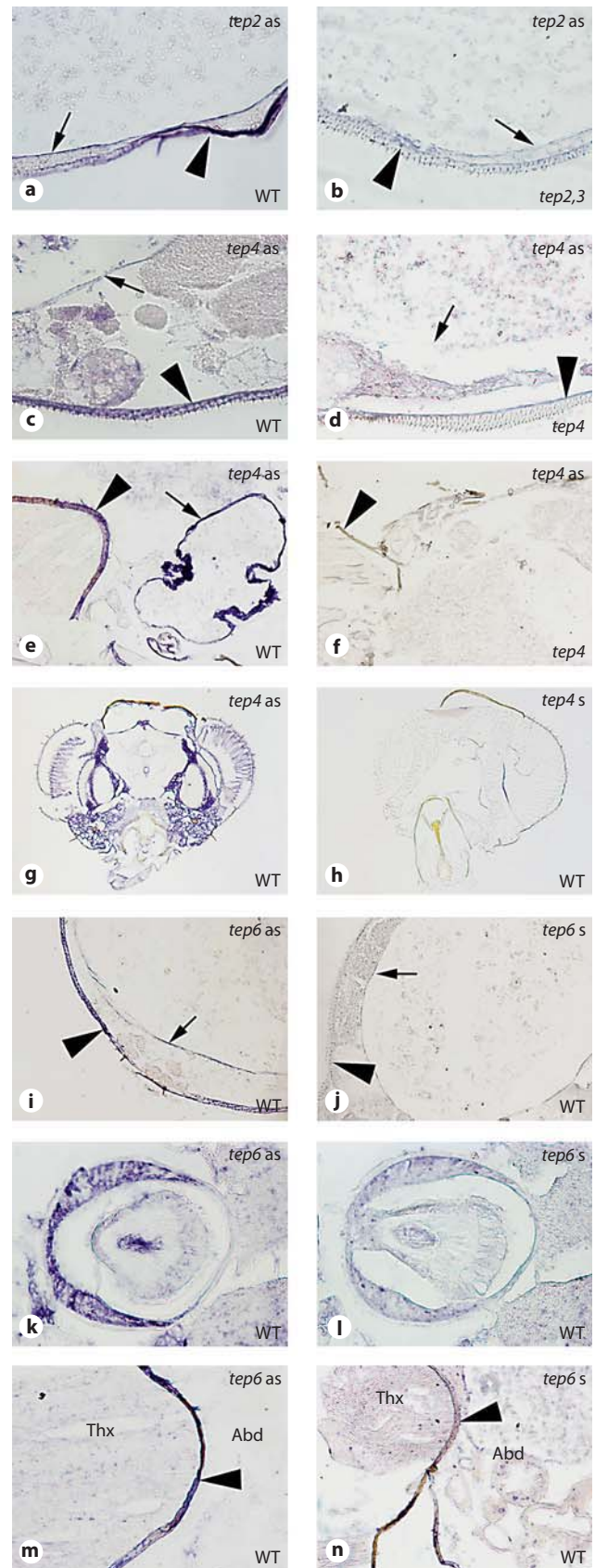
**Fig. 4.** Expression of *Tep* genes in larvae. Whole-mount ISH on late third instar larvae. **a–d** *Tep2*. **e–j** *Tep4*. **k–n** *Tep6*. Left-hand panels = *Tep* gene expression; right-hand panels = controls. The probe used for ISH is indicated on the top right corner of each panel, whereas the genotype is indicated on the bottom right corner. The following tissues are shown: hemocytes (**a, b, e, f, k, l**); fat body (**c, d, g, h**); lymph glands (arrow), central nervous system, and imaginal discs (arrowhead; **i, j, m, n**). For *Tep6*, we used the sense probe on wild-type (WT) larvae as control, as *Tep6* mutants (*Mcr*) are lethal.

humoral and the cellular responses, respectively [6], the distributions of *Tep1*-GFP, *Tep2*, *Tep3*, *Tep4*, and *Tep6* mRNA matched the main tissues involved in larval immunity. Interestingly, *Tep6* is expressed in all imaginal discs (fig. 4m, n).

In adults, we found that the digestive tract was a major site of expression of *Tep* genes. The epithelium of the crop, a diverticulum of the digestive tract that is used to store food, expressed *Tep2*, *Tep3*, *Tep4*, and *Tep6* (fig. 5a–f, i, j; online suppl. fig. S2). The proventriculus, the valve-like structure that guards the midgut entrance, was found to be positive for *Tep1*-GFP and *Tep6* expression (fig. 5k, l; online suppl. fig. S3). However, we did not detect the expression of these *Tep* genes in the midgut itself. In addition to the epithelia of the digestive tract, we found that the epidermis underlying the cuticle in the abdominal part was a major site for *Tep* gene expression. Interestingly, *Tep2*, *Tep3*, *Tep4*, and *Tep6* expression was restricted to the ventral side of the abdominal epidermis (fig. 5a–d, i, j; online suppl. fig. S2). In addition, *Tep4* and *Tep6* expression was detected in the mesophragma, the structure found at the border between thorax and abdomen and to which flight muscles are attached posteriorly (fig. 5e, f, m, n). Surprisingly, in contrast to larvae, no expression could be detected in the abdominal fat body (before or after immune challenge) of adult flies, except in the fat body of the head region where *Tep4* (fig. 5g, h) was strongly induced. Interestingly, upon a septic injury, the expression pattern of *Tep2*, *Tep4*, and *Tep6* remained unchanged in adults (data not shown).

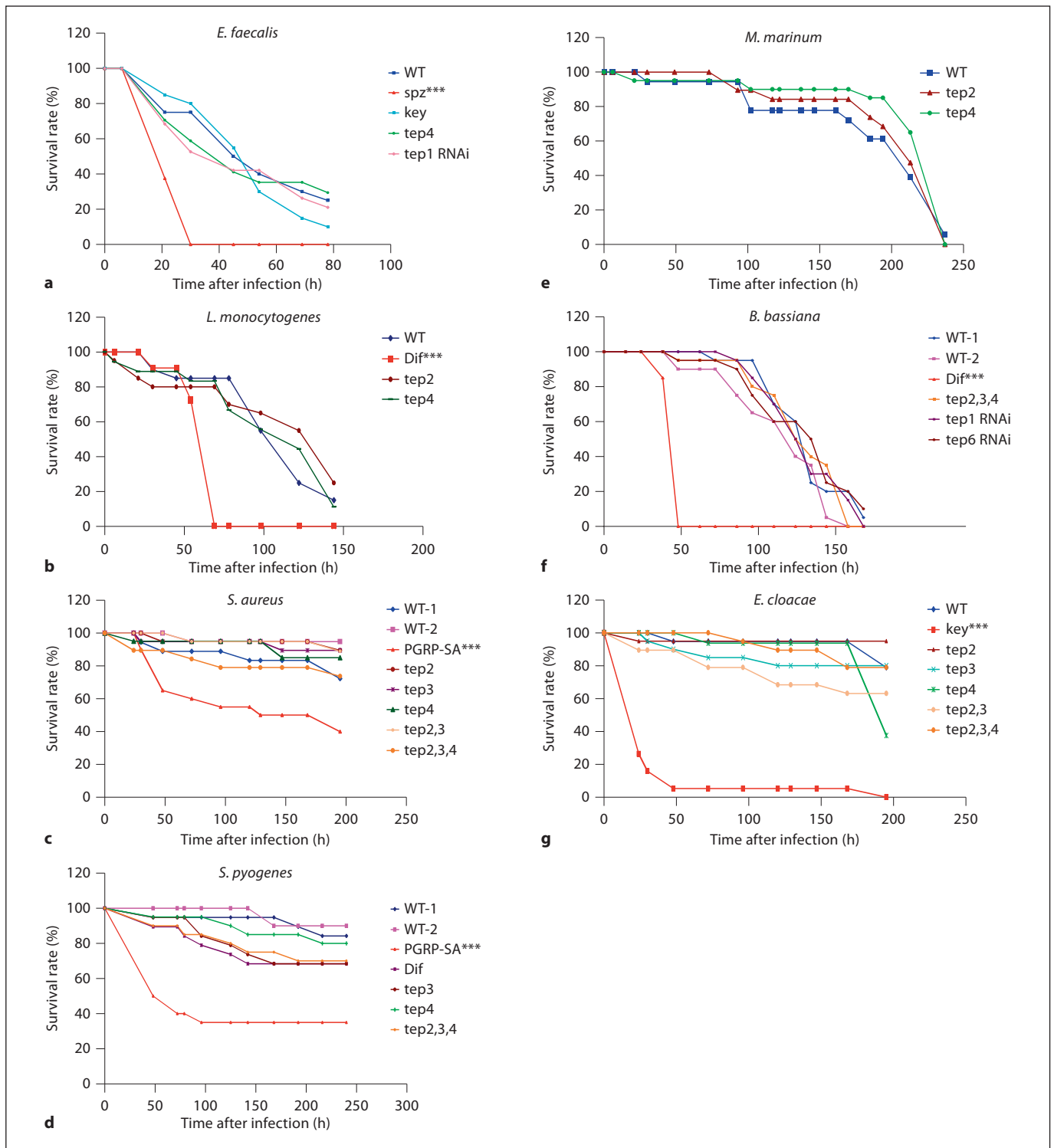
#### *TEPs Appear Not to Be Required for D. melanogaster Survival upon Microbial Challenge*

Since most *Tep* genes are expressed in hemocytes (fig. 4a, b, e, f, k, l) and are predicted to act as opsonins [12], we decided to investigate their function in the defense against pathogens. We thus challenged *Tep* mutants with different microbes introduced in the hemocoel by a



**Fig. 5.** Expression of *Tep* genes in adults. ISH on paraffin-embedded longitudinal and transverse sections. **a, b** *Tep2*. **c–h** *Tep4*. **i–n** *Tep6*. Left-hand panels = *Tep* gene expression; right-hand panels = controls. The probe used for ISH is indicated on the top right corner of each panel, whereas the genotype is indicated on the bottom right corner. The following tissues are shown: crop (arrows) and hypoderm (arrowheads; **a–f, i, j**); head (**g, h**); proventriculus (cardium; **k, l**); mesophragma (arrowheads; **e, f, m, n**). For *Tep6*, we used the sense probe on wild-type (WT) flies as control, as *Tep6* mutants (*Mcr*) are lethal.





**Fig. 6.** *Tep* mutants survive as wild-type (WT) flies to different types of infection. Survival experiments after distinct challenges in the septic injury model are presented and are representative of at least two independent experiments. The appropriate controls for the different microbes have been used: Gram-positive bacteria, fungi: mutants of the Toll pathway [*Dif*, *spätzle* (*spz*), *PGRP-SA*]

and IMD pathway: *kenny* (*key*). None of the *Tep* mutants shows a reproducible susceptibility or resistance to infection phenotype, either in homozygous or hemizygous conditions. We used the log-rank test to determine the significance between wild-type and mutant survival curves. \*\*\*  $p < 0.001$  was the only constantly measured  $p$  value in several experiments.



**Fig. 7.** *Tep* mutants display normal phagocytosis activity. Adult *Drosophila* carcasses from WT (**a**, **c**) and *Tep* 2,3,4 mutant (**b**). pHrodo™ *E. coli* BioParticles® produce a red fluorescence when exposed to an acidic environment such as that present in the

phagosome. It labeled sessile hemocytes (arrowheads; **a**, **b**) and pericardial cells (asterisk; **a–c**). Injection of latex beads, prior to pHrodo *E. coli* BioParticles injection (WT + Lxb), blocked phagocytosis. Note that the pericardial cells were unspecifically labeled.

septic injury (fig. 6). Survival experiments were performed in adult flies with either Gram-positive (*S. pyogenes*, *S. aureus*, *E. faecalis*, *L. monocytogenes*, and *M. marinum*) or Gram-negative bacteria (*E. coli* and *E. cloacae*). *S. pyogenes* was chosen because patients deficient in the complement system are commonly suffering from recurrent pyogenic infections. *M. marinum* was used as *Tep2* and *Tep4* are the most strongly induced genes in this infection model [11]. As phagocytosis plays a critical role in the clearance of *E. faecalis* and *S. aureus* [25, 26, Nehme et al., submitted], infections with these bacteria are more likely to reveal an involvement of TEPs in phagocytosis.

Our results showed that *Tep1*-, *Tep2*-, *Tep3*-, and *Tep4*-deficient flies were not more susceptible to bacteria than wild-type flies (fig. 6a–e, g, and data not shown). Additionally, we studied the survival rate of *Tep* mutants after a fungal infection using spores from the entomopathogenic fungus *B. bassiana* [22] and we found that *Tep* mutant flies for either *Tep1* (RNAi), *Tep2*, *Tep4*, or *Tep6* (RNAi) survive as well as wild-type flies to *B. bassiana* natural infection (fig. 6f). We also observed a normal resistance to microbial infections in flies hemizygous for *Tep2*, *Tep3*, and *Tep4*. Collectively, these results showed that none of the tested *Tep* genes is required on its own for the defense against the bacterial and fungal infections we have tested.

Given (i) the expression pattern of multiple *Tep* genes in the hemocytes and the epidermis underlying the cuticle; (ii) that TEPs are secreted into the hemolymph, and (iii) that *Tep1*, *Tep2*, and *Tep4* belong to the same phylogenetic group, we hypothesized that the function(s) of TEPs in the hemolymph might be redundant, at least to some extent. To test this hypothesis, we generated flies doubly

mutant for *Tep2* and *Tep3* or triply mutant for *Tep2*, *Tep3*, and *Tep4*, and then assessed their susceptibility to septic injuries with different microbes (*E. cloacae*, *S. aureus*, *E. faecalis*, *L. monocytogenes*, *S. pyogenes*, and *B. bassiana*). These compound mutants resisted normally to these infections (fig. 6c, d, g, and data not shown). Accordingly, a phagocytosis assay performed on compound *Tep2*, *Tep3*, and *Tep4* mutant flies failed to reveal any qualitative defects in the activity of sessile hemocytes (fig. 7).

## Discussion

### Do TEPs Function in *D. Melanogaster* Innate Immunity?

The finding that *Tep2*, *Tep3*, or *Tep4* mutant flies resist the microbial infections we have tested in wild-type flies for example is somewhat unexpected because: (i) it was shown in *Drosophila* S2 cells that TEP2 and TEP3 are required for the phagocytosis of *E. coli* and *S. aureus*, respectively [12], and (ii) phagocytosis in flies is essential to defend against *S. aureus* and *E. faecalis* [25, 26, Nehme et al., submitted]. It has been shown that TEP6 binds to *C. albicans* and is required for its phagocytosis by cultured S2 cells [12]. We did not assess the survival of *Tep6* mutant flies to this infection as null mutants are larval lethal.

According to the phylogenetic analysis described above, we speculate that *Tep1* and *Tep2* are likely to act redundantly. Thus, the deletion of both genes will be required to test this hypothesis. The combinatorial role of TEPs remains therefore to be wholly investigated.

Most *Tep* genes are constitutively expressed in barrier epithelia and especially in weak points of the cuticle

(fig. 5). Thus, these proteins may serve to anticipate infections by pathogens attacking through the cuticle, i.e. some entomophthorales fungi, or microbes transmitted by blood-sucking mites or wasps. Thus, we may not have tested the relevant pathogens.

Because many *Tep* genes (fig. 3, and data not shown) are expressed in portions of the digestive tract of adult flies and larvae, it will be of interest in the future to combine the inactivation of these *Tep* genes with an oral infection model to unravel more subtle or physiological functions of TEPs.

#### *Tep* Genes during Evolution

The failure to observe an innate immunity phenotype for *D. melanogaster* *Tep* mutants contrasts the results obtained with mosquito *A. gambiae* TEP1 [8, 9]. Nevertheless, analysis of the phylogeny of *Tep* genes reveals that *Tep1* from *Anopheles* belongs to the group of *Tep* genes (group C) that is restricted to mosquito species. The founder gene of group C may have arisen by duplication from *Anopheles* *Tep13* (group A) or *Tep15* (group B) and selected for its adaptation to the mosquito life cycle and ecology (fig. 1). Mosquitoes are blood-sucking insects that have an aquatic life during their larval stage and an aerial life during their imaginal stage. We did not find any *Tep* genes belonging to group C in other blood-sucking pests present in our phylogenetic analysis panel, such as *I. scapularis* and *P. humanus*. It is thus unlikely that the blood meal provides on its own a significant selective pressure for the appearance or maintenance of group C *Tep* genes. However, the Anophelinae *A. gambiae* and the Culicinae *A. aegypti* and *C. pipiens* may have evolved more recently in blood-sucking insects than the chelicerate *I. scapularis* and the Phthirapter *P. humanus*. Therefore, group C-related *Tep* genes might reflect a more recent adaptation to blood feeding. It will be of interest to analyze the genome of the tsetse fly *G. morsitans* when released. Another possibility is that the appearance of this *Tep* group mirrors an adaptation to the dual aquatic and aerial biotopes that mosquitoes encounter during their life cycle. Again, the genomes of insects displaying a similar life cycle such as the Odonata *Libellula* may provide the answer to this question.

Additionally, our phylogenetic analysis shows that *Tep6* is highly conserved within Drosophilidae, which suggests that the function of this gene is under a strong selective pressure. Also, we found that *Tep6* is expressed in tissues required for metamorphosis (e.g. imaginal discs). This may reflect a role for *Tep6* either during metamorphosis (or development), which may account for the

lethality of *Tep6* mutants. To a lower extent, *Tep3* genes are also significantly conserved within the Drosophilidae group. This suggests that the function of *Tep3* is highly selected for. It is of interest to note that the *Tep3*-related genes from group B are also highly conserved in ecdysozoa and lophotrochozoa (fig. 1a). This further suggests that the function of *D. melanogaster* *Tep3* is likely to be (i) reminiscent of the *Tep* ancestral function, and (ii) conserved among protostomes.

In conclusion, our study defines three clusters of *Tep* genes among protostomes. Each of these clusters most probably reflects a particular biological function of TEPs. We failed to identify a role for *Tep1*, *Tep2*, *Tep3*, and *Tep4* in *D. melanogaster* innate immunity using a septic injury model or a 'natural' fungal infection. We cannot exclude that the immune function of TEPs is masked by other defense mechanisms. Interestingly, we found that the expression pattern of *Drosophila* *Tep* genes is compatible with a defense function against pathogens in barrier epithelia [27–29]. Additionally, a population genetic study predicted that the rapid evolution of *D. melanogaster* TEP1 and TEP2 is likely to be the signature of host-pathogen co-evolution, indicating a specific role of TEPs against a restricted subset of pathogens [30]. Thus, it is likely that the investigation of infection models with natural pathogens such as *Serratia marcescens*, *Pseudomonas entomophila*, *Providencia sneebia*, *Providencia burhodogranariaea*, *Tubulinosema ratisbonensis* (microsporidia), or *Heterorhabditis bacteriophora* (nematode symbiotically associated with the entomopathogenic bacterium *Photorhabdus luminescens*) is likely to yield novel insights into the potential immune functions of *Tep* family genes [31–36].

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#### Disclosure Statement

We report no conflict of interest.

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