RESEARCH ARTICLE

A novel mode of induction of the humoral innate immune response in Drosophila larvae

Hiroyuki Kenmoku1,*, Aki Hori1,2,*, Takayuki Kuraishi1,3,4,5,*,†,§ and Shoichiro Kurata1,*

ABSTRACT

Drosophila adults have been utilized as a genetically tractable model organism to decipher the molecular mechanisms of humoral innate immune responses. In an effort to promote the utility of Drosophila larvae as an additional model system, in this study, we describe a novel aspect of an induction mechanism for innate immunity in these larvae. By using a fine tungsten needle created for manipulating semiconductor devices, larvae were subjected to septic injury. However, although Toll pathway mutants were susceptible to infection with Gram-positive bacteria as had been shown for Drosophila adults, microbial clearance was not affected in the mutants. In addition, Drosophila larvae were found to be sensitive to mechanical stimuli with respect to the activation of a sterile humoral response. In particular, pinching with forceps to a degree that might cause minor damage to larval tissues could induce the expression of the antifungal peptide gene Drosomycin; notably, this induction was partially independent of the Toll and immune deficiency pathways. We therefore propose that Drosophila larvae might serve as a useful model to analyze the infectious and non-infectious inflammation that underlies various inflammatory diseases such as ischemia, atherosclerosis and cancer.

KEY WORDS: Innate immunity, Drosophila, Larvae

INTRODUCTION

Drosophila adults have been used as a leading model organism to investigate molecular mechanisms of innate immunity (Lemaître and Hoffmann, 2007; Buchon et al., 2014) since it was first demonstrated in 1996 that the Toll pathway, which was initially characterized as an essential pathway for dorsoventral patterning in Drosophila embryos (Anderson et al., 1985a,b), was required for the induction of the antifungal peptide gene Drosomycin (Drs) upon fungal infection (Lemaître et al., 1996). In particular, Drosophila adult models have contributed to identifying genes required for the humoral innate immune responses and for the production of antimicrobial peptides (AMPs) and melanization factors (Lemaître et al., 1995; Rämét and Hultmark, 2014). In Drosophila adults, AMP induction upon challenge with microbes is controlled by two distinct signaling pathways, the Toll and immune deficiency (IMD) pathways (Lemaître and Hoffmann, 2007; Valanne et al., 2011; Myllymäki et al., 2014). The Toll pathway is required for the induction of Drs and for survival following systemic infection with Gram-positive bacteria or fungi (Ferrandon et al., 2007). Specifically, the recognition of lysine-type peptidoglycans or β-glucans from microbes by the PGRP-SA/GNBPI complex or by GNBPI3 in the hemolymph activates modular serine protease (ModSP), followed by activation of Spätzle (Spz)-processing enzyme and cleavage of Spz, a protein ligand of the Toll receptor (Gottar et al., 2002, 2003, 2006; Jang et al., 2006; Buchon et al., 2009b). In addition, so-called ‘danger signals’ also activate the Toll pathway through the protease Persephone (Psh). For example, exogenous danger signals such as PR1 secreted from pathogenic fungi, as well as endogenous danger signals generated in apoptosis-deficient mutants, lead to the activation of Psh and subsequent processing of Spz (Chany et al., 2008; Ming et al., 2014; Otaba et al., 2014). The active form of Spz induces conformational changes in the Toll receptor, activates Toll intracellular signaling (Kanoh et al., 2015b) and ultimately leads to the nuclear translocation of nuclear factor-kappa B (NF-κB) proteins Dif and Dorsal, inducing the expression of antimicrobial peptide genes including Drs (Lindsay and Wasserman, 2014). Conversely, the IMD pathway recognizes diaminopimelic acid-type peptidoglycans derived from Gram-negative bacteria via peptidoglycan recognition protein (PGRP)-LC and PGRP-LE (Kleino and Silverman, 2014). These receptors facilitate downstream signaling via the adaptor protein IMD, activate the NF-κB protein Relish, and induce the expression of antimicrobial peptides such as Diptericin (Dpt) (Paquette et al., 2010). Notably, these pathways are essentially characterized in Drosophila adults.

In contrast, Drosophila larvae have been largely utilized for dissecting cellular immune responses, particularly for nematode and wasp infections (Paddibhatla et al., 2010; Arefin et al., 2015; Kucerova et al., 2015; Hillyer, 2016). Insect hemocytes, representing blood cells, are composed of three cell types: plasmatocytes, crystal cells, and lamellocytes. These play central roles in cellular immunity by phagocytosing bacteria (plasmatocytes), involvement in the melanization process (crystal cells) and forming capsules around wasp eggs, a process referred to as encapsulation (lamellocytes) (Honti et al., 2014; Gold and Brückner, 2015; Parsons and Foley, 2016). For example, recent studies have begun to unravel the complex encapsulation processes by using Drosophila larvae upon infection with parasitoid wasps such as Leptopilina boulardi (Kari et al., 2016). In addition, the fat body, an immune-responsive organ in flies functionally resembling the mammalian liver, expresses edin and utilizes Toll signaling to control the numbers of plasmatocytes (Schmid et al., 2014; Vanha-aho et al., 2015). Finally, JAK-STAT signaling in somatic muscles is important for inducing the encapsulation reaction and controls the number of circulating lamellocytes (Yang et al., 2015).
By contrast, only a handful of studies have been published related to use of the Drosophila larval model of bacterial infection to analyze humoral immune responses (Ferrandon et al., 1998; Manfruelli et al., 1999; Ligoyygakis et al., 2002; Shia et al., 2009; Yamamoto-Hino et al., 2015; Yamamoto-Hino and Goto, 2016). Because these studies implicate intriguing differences in terms of the induction mechanisms of AMPs between larvae and adults, a larval model might thus have the potential to identify novel molecular mechanisms. However, it is possible that the limited numbers of publications on larval bacterial infection might partly be due to technical difficulties in the manufacture of uniform tungsten wires sharpened by electrolysis and their use in introducing infections (Romero and Lemaitre, 2008) without causing severe damage that leads to the death of the larvae. Consistent with this likelihood, the survival and colony-forming assays upon systemic infection in larvae have been seldom reported. Here, we present a method to perform larval infection using a tungsten needle provided by a manufacturer that produces pins for testing semi-conductor devices. By using this uniform and solid needle, we were able to successfully perform and investigate bacterial infection in Drosophila larvae. In addition, we found that mechanical stimuli generated by pinching larvae with forceps resulted in the sterile induction of an antimicrobial peptide, providing a novel model for non-infectious activation of the humoral innate immune response.

**RESULTS**

**The Toll pathway is required for survival against Gram-positive bacterial infection in larvae but not for bacterial removal**

To easily and consistently perform infection using third instar larvae, we employed a fine tungsten needle used for the examination of semiconductor devices. With this needle, over 80% of larvae were able to survive following a clean injury in the wild type and in Toll pathway and IMD pathway mutants (Fig. 1A). By pinching larvae with a needle dipped into a pellet of Gram-positive bacteria Staphylococcus saprophyticus, we found that Toll pathway mutants were susceptible to the infection (Fig. 1B), although the number of bacteria in the infected whole mutant larvae after any time point was similar to that in the wild type (Fig. 1C). These results suggest that the Toll pathway is dispensable for bacterial clearance in larvae, showing a sharp contrast to the results from Drosophila adults in which the Toll pathway is required for the removal of bacteria upon Gram-positive bacterial challenge. Notably, although the induction of the antifungal peptide gene Drs was slightly lower in Toll pathway mutants than in wild-type larvae, substantial induction of Drs still remained in the mutants (Fig. 1D), consistent with the results of Manfruelli et al. (1999).

We next challenged larvae with Gram-negative bacteria using the needle. Fig. 1E and F show that IMD mutant larvae were not sensitive to infection with Ecc15, although the induction of the antibacterial peptide gene Dpt was almost abrogated in the mutant. From these results, we conclude that survival, AMP expression and bacterial number upon bacterial infection by septic injury with a tungsten needle could be consistently measured in Drosophila larvae, and that the role of the Toll pathway was somewhat different during this process compared with the adult infection model.

**Pinching by forceps induces the expression of AMP in larvae**

We found that clean injury with the needle induced the expression of Drs and Dpt (Fig. 2A,B). Furthermore, even pinching larvae using forceps, a normal means of handling larvae, caused strong Drs induction (Fig. 2A). Time-course experiments showed that Drs expression was induced from 2 h, maximized at 4 h and continued to 12 h (Fig. 2C). The level of Drs after eclosion was not increased compared with the level in untreated flies (Fig S4A). After pinching, 10% of larvae showed small melanized spots (Fig. 2D), although extremely weakly pinched larvae did not show melanization and the level of Drs induction was marginal (Fig. S4B), implying that pinching might cause minor injury in larval tissues. Next, we examined which tissues exhibited Drs expression. Fig. 2E shows that Drs reporter larvae exhibited GFP signals in the whole fat body and that the position of pinching was not connected with Drs induction. Consistent with this result, quantitative real-time-polymerase chain reaction (real-time qPCR) analysis showed that the induction of Drs was detected in the fat body dissected out from other tissues (Fig. 2F). These results indicate that Drs is induced in the fat body upon pinching with forceps.

As Drosophila possess commensal bacteria (Kuraishi et al., 2013), Drs induction by pinching might be caused by such infections. To assess this possibility, germ-free larvae (Fig. 2G) were used for pinching experiments. Fig. 2H shows that the level of induction of Drs in germ-free larvae was not reduced compared with that in conventionally reared larvae, indicating that Drs expression is steriley induced by pinching with forceps. Next, we performed microarray analysis using pinched larvae in order to examine whether Drs was uniquely induced by pinching or whether other defense response genes that respond to infection in adults (De Gregorio et al., 2001, 2002) were also induced. We found that in addition to Drs, several immune-related genes such as IM1, IM3, IM10 and Attacin were induced over 10-fold upon pinching with forceps (Table 1). In addition, stress responsive genes such as ToxA, ToxA and TotC were induced in the larvae. This result suggests that pinching larvae with forceps induces a humoral innate immune response that is similar to that observed in systemic infection in adults. We also noticed that a number of chitin metabolic genes were also downregulated upon pinching (Table 2).

**Toll pathway genes contribute to the induction of Drs upon pinching with forceps**

Next, we asked which signaling pathway is involved in the induction of Drs upon larval pinching. Real-time qPCR analysis showed that the level of induction in the spz mutant or dMyd88 mutant was approximately half that of the wild-type larvae (Fig. 3A). In contrast, the induction of Drs was comparable to that in the wild-type in the Dif mutant, or psh and modSP double mutants (Fig. 3A). These results suggest that certain Toll pathway components are partly required for the induction of Drs upon pinching. We next investigated IMD pathway mutants and found that larvae of the pgrp-le and pgrp-le double mutant, imd mutant or relish mutant exhibited normal Drs induction after pinching (Fig. 3B). Furthermore, the level of induction of Drs in the double mutant larvae for imd and spz, or for relish and spz was almost the same as that in the spz single mutant (Fig. 3D), suggesting that the Toll and IMD pathways did not have a redundant role in pinching-induced Drs expression. We further investigated the involvement of the JAK-STAT, JNK, p38, dFOXO and pro-PO pathways, all of which suggested a role for AMP induction or host defense under certain conditions (Kim et al., 2002; Buchon et al., 2009a; Becker et al., 2010; Chen et al., 2010; Binggeli et al., 2014; Parisi et al., 2014). Inhibition of the JAK-STAT pathway by using an upd2 and upd3 double mutant did not reduce the induction of Drs in pinched larvae (Fig. 3C), implying that the JAK-STAT pathway may be dispensable for Drs induction. Similarly, the normal Drs induction observed upon pinching in larvae with an eiger mutation or c564-GAL4-driven expression of a dominant negative form of
Bsk implied that there was no requirement of the JNK pathway in Drs induction (Fig. 3E). The level of Drs induction was also same in wild-type larvae as in the larvae of p38a, p38b and p38c (Fig. 3F), dfoxo (Fig. 3G) and PPO (Fig. 3H) mutants, indicating that the p38, dFOXO and pro-PO pathways played no role in the induction of Drs following larval pinching with forceps.

Sensory neurons and hemocytes are dispensable for the induction of Drs upon pinching larvae with forceps

When pinching larvae with forceps, we touched the larval cuticle under which the web of sensory neurons exists, prompting us to examine the role of sensory neurons in pinching-induced Drs expression. We first ablated sensory neurons by expressing the
apoptosis-inducing genes reaper and hid with a pan-sensory neuron 
GAL4^{109(2)80} driver (Fig. 4A, Fig. 5S) or class IV sensory neuron 
ppk-GAL4 driver. Fig. 4A shows that the level of induction of 
Drs in larvae with sensory neurons ablated by either driver was the same as 
that in the wild type. However, no effect was again observed 
on Drs induction when neurotransmission was suppressed 
(Fig. 4B). Conversely, we then monitored Drs expression using 
larvae in which the sensory neurons were artificially activated by 
expressing the dTrpA1 ion channel (Hamada et al., 2008). Fig. 4C 
shows that Drs was not induced in the activated larvae without 
pinching. These results collectively suggest that sensory neurons 
are not involved in the induction of Drs upon larval pinching with 
forceps.

As Spz has been suggested to be secreted from hemocytes (Shia 
et al., 2009), we tested the role of hemocytes in the induction of 
Drs upon pinching. We observed that hemocyte-specific expression of 
reaper and hid mediated by using a hmlA-GAL4 driver effectively 
ablated hemocytes in larvae (Fig. 4D). Using these larvae, we next 
examined the induction of Drs upon pinching and found that no 
difference could be detected between wild-type and hemocyte-
ablated larvae with respect to the level of Drs induction (Fig. 4E). 
Consistent with this, inhibition of the phagocytic function of
hemocytes by expressing Shibire (Awasaki and Ito, 2004) had no effect on the induction of Drs upon pinching (Fig. 4E). These results suggest that hemocytes are dispensable for the induction of Drs after pinching with forceps.

**DISCUSSION**

In this study, we present a method by which systemic bacterial infection can be performed easily and consistently in *Drosophila* larvae, thus providing another genetically tractable model to decipher infectious diseases. In this model, we show that the role of the Toll pathway in resistance against systemic infection in *Drosophila* larvae differs from that in adults to a certain extent. Specifically, the Toll pathway is likely to be required for tolerance of *Drosophila* larvae possessing a novel mode of induction of the humoral innate immune response that might represent a good model for studying the mechanisms underlying sterile inflammation. Although we demonstrated that Dif, a *Drosophila* NF-κB essential for the induction of Drs upon systemic infection in adults, was not involved in pinching-induced Drs expression in larvae, we could not rule out the possible involvement of NF-κB in transactivating Drs expression, as we were unable to examine the redundant role of the other NF-κB proteins, Dorsal and Relish, because of the unavailability of viable lines. The dependency on NF-κB remains a question to be solved in future studies.

While performing these infection studies, we serendipitously found that the humoral innate immune response is activated in *Drosophila* larvae by modest mechanical stimuli; i.e. by pinching larvae with forceps, as they are commonly handled. AMP expression induced by pinching in larvae is sterile and partially independent from known innate immune signaling; these conclusions are supported by the following evidence: (1) the induction of Drs was observed in germ-free larvae upon pinching; (2) substantial induction of Drs remained in double mutants for the Toll and IMD pathways; (3) normal induction of AMPs; the latter being consistent with suggestions from a previous study (Manfruelli et al., 1999). Drs, an antimicrobial peptide whose expression is under the control of the Toll pathway, is strongly induced upon Gram-positive bacterial infection, although Drs is only active against fungi but not bacteria (Fehlbaum et al., 1994). One possibility to explain the former discrepancy might be that certain genes induced upon infection, including Drs, might function in conjunction to confer resistance and tolerance to adults and larvae, albeit with as-yet unknown mechanisms. This point should be elucidated in future research.

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### Table 1. Top 20 genes upregulated after pinching larvae with forceps

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene symbol</th>
<th>Gene ontology biological process</th>
<th>Pinching</th>
<th>Clean injury</th>
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<tr>
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<td>Defense response</td>
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<td>122.7</td>
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<td>Defense response</td>
<td>85.4</td>
<td>118.3</td>
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<td>Response to stress</td>
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<td>Defense response</td>
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<td>Defense response</td>
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<td>TotB</td>
<td>Response to stress</td>
<td>10.7</td>
<td>24.5</td>
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At 4 h after pinching or clean injury to *yw* larvae, expression profiles were analyzed by DNA microarray using whole larvae. The table shows the top 20 genes that were upregulated upon pinching. Probe set IDs, gene symbols, Gene ontology biological processes, and the fold change in gene expression compared with no treatment (0 h) are indicated.

### Table 2. Top 20 genes downregulated after pinching larvae with forceps

<table>
<thead>
<tr>
<th>Probe set ID</th>
<th>Gene symbol</th>
<th>Gene ontology biological process</th>
<th>Pinching</th>
<th>Clean injury</th>
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At 4 h after pinching or clean injury to *yw* larvae, their expression profiles were analyzed by DNA microarray using whole larvae. The table shows the top 20 genes that were downregulated upon pinching. Probe set IDs, gene symbols, Gene ontology biological processes, and the fold change in gene expression compared with no treatment (0 h) are indicated.
Fig. 3. See next page for legend.
have established a sterile inflammation model in larvae (Shaukat et al., 2015). Ming et al. (2014) established a larval model for sterile AMP induction using a caspase mutant. They showed that the induction of Drs is solely dependent on Spz and Persephone, suggesting that the molecular mechanism of Drs induction in this mutant is different from our ‘pinching’ model. In addition, Hauling et al. (2014) and Parisi et al. (2014) reported that tumors can induce the expression of AMPs. Parisi et al. (2014) demonstrated that the induction of Drs is dependent on eiger and spz, both of which are not essential for our pinching-induced expression of Drs.

Furthermore, Kanoh et al. (2015a) showed that Drosophila larvae possess another intrinsic ligand for the Toll receptor in addition to Spz, although its molecular nature has not yet been identified. Together, these reports suggest that Drosophila larvae possess multiple modes of induction of AMPs in response to various sterile stimuli that activate innate immunity.

In the current study, we showed that pinching stimuli can induce AMP expression; however, the physiological relevance of this phenomenon has not yet been elucidated. The larvae of Drosophila melanogaster in the wild are expected to be exposed to serious likelihood of attack by parasitoid wasps. Thus, mechanical stimuli might be considered as a potential infectious danger, suggesting that even small injuries resulting from oviposition might be able to activate AMP expression. Consistent with this, Schmid et al. (2014) recently showed that overactivation of Toll signaling could provoke a cellular immune defense that has potential importance in the response to wasp infection.

In conclusion, we demonstrate in this study that Drosophila larvae represent a suitable model in which to perform microbial infection by using a fine and uniform tungsten needle and to assess sterile induction of the humoral immune response by pinching larvae with forceps. In particular, because pinching-induced AMP expression is likely to be dependent on an as-yet uncharacterized phenomenon has not yet been elucidated. The larvae of Drosophila melanogaster in the wild are expected to be exposed to serious likelihood of attack by parasitoid wasps. Thus, mechanical stimuli might be considered as a potential infectious danger, suggesting that even small injuries resulting from oviposition might be able to activate AMP expression. Consistent with this, Schmid et al. (2014) recently showed that overactivation of Toll signaling could provoke a cellular immune defense that has potential importance in the response to wasp infection.

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molecular mechanism, our model might be useful to decipher the complex mechanisms that regulate sterile inflammation, which has considerable importance for the treatment of inflammatory diseases in humans such as ischemia, atherosclerosis and cancer.

**MATERIALS AND METHODS**

**Fly stocks and maintenance**

*Drosophila* stocks were maintained in standard corn meal-yeast agar medium vials at 25°C. Oregon R, w, and y flies were used as wild-type controls. As Toll pathway mutants, *spz* mutants (Morisato and Anderson, 1994), *spz* 

\[ \text{spz}^{\text{ABH1}} \]

(described below), mod5P3P7; psh1; mod5P6 (a gift from Dr Bruno Lemaître) (Chamy et al., 2008; Buchon et al., 2009b), for *Dif* (described below), *Dif* (a gift from Drs Jean-Marc Reichhart and Dominique Ferrandon) (Rutschmann et al., 2000) and dm88/3 (a gift from Dr Jean-Luc Imler) (Chararti et al., 2003) were used. As IMD pathway mutants, *PGR-LE12;PGR-LEC12* (Gao et al., 1999; Takehana et al., 2004), *imd* (a gift from Dr Bruno Lemaître) and *Relish* (Lemaître et al., 1995; Hedengren et al., 1999) were used. The following mutants were used: *hsk* (Bloomington Drosophila Stock Center (BDSC), 3088), *eiger* (a gift from Dr Masayuki Miura) (Ikagi et al., 2002), *spz*; mod5P3P7 (a gift from Dr Bruno Lemaître) (Osman et al., 2013), *PPO1*; *PPO2*; *PPO3* (a gift from Dr Bruno Lemaître) (Binggeli et al., 2014; Dudzic et al., 2015), *dfoxo* and *dfoxo* (a gift from Dr Marc Tatar) (Jünger et al., 2003; Weber et al., 2005), as well as *p3813*, *p3816* and *psps* (a gift from Dr Bruno Lemaître) (Davis et al., 2008; Chen et al., 2010; Chakrabarti, et al., 2014).

The following transgenic flies were used: *Dr-GFP Dpt-lacZ* (a gift from Dr Dominique Ferrandon) (Jung et al., 2001), *spz-GAL4, GAL4* (Osborne et al., 1997) we happened to find that the *spz* (a gift from Drs Jean-Marc Reichhart and Dominique Ferrandon) (Jung et al., 2001), *casp* (described below), *Dif*-genome-PCR-F1-9: 5′-CTT CCG AGG TAT TAT TAT CCG CC-3′; *spz-ex1_1_1 F*: 5′-CTT CCG AGG TAT TAT TAT CCG CC-3′; *spz-ex1_1_1 R*: 5′-AAA CCC GAT CCG CAA ACC TAC GCC TGC-3′; *Dif-ex1-1-1 F*: 5′-CTT CGC AGG TAT TAT TAT CCG CC-3′; *Dif-ex1-1-1 R*: 5′-AAA CCC GAT CCG CAA ACC TAC GCC TGC-3′; *Dif-ex2-1-1 F*: 5′-CTT CGC AGG TAT TAT TAT CCG CC-3′; *Dif-ex2-1-1 R*: 5′-AAA CCC GAT CCG CAA ACC TAC GCC TGC-3′. The constructed vector was used to generate the 6-8-spz-GRN4 line, by using the y* w 71-23; P[CaryP] attP2 (BDSC, 8622) line (performed in BestGene). The 6-8-spz-GRN4 line was crossed to nos-Cas9 (National Institute of Genetics, CAS-0001) as described in Kondo (2014) to generate candidate deletion lines. Genomic DNAs of each candidate mutant were screened by PCR to check for the deletion (Fig. 2S) using the following primers: *spz-Fw*: 5′-GGA ACT GCT AGA ACA ACT ATG GA-3′; *spz-Rv*: 5′-CAG TAA CAC CAG CTA CCA GT-3′; *Drs-Fw*: 5′-GTG ACT GCA GTAT TCA TTA TTT G-3′; and *Drs-Rv*: 5′-GTG GGT CCG GAA CAT TAG GG-3′. One line, *spz*-8H, was found to have a 350 bp deletion that includes the start codon (Fig. 2F); in addition, the line, *Drs*-17-17 was found to have a 210 bp deletion that includes the start codon (Fig. 2F); thus, we used these lines as *spz* or *Drs* null mutants, respectively.

To prove neural activity, *ppk-GAL4* or *GAL4* (Osborne et al., 1997) was crossed to *UAS-mCD8; GFP* was crossed to *UAS-dTrpA1* and maintained at 18°C until they had developed into third instar larvae. In *ppk-GAL4*, third instar larvae were incubated at 29°C prior to assessment. In *GAL4* (Osborne et al., 1997) third instar larvae were incubated in a water bath at 37°C twice for 2 min (10 min intervals at 25°C), then maintained at 25°C for 4 h and used for assays.

To inhibit neural activity, *GAL4* (Osborne et al., 1997) was crossed to *UAS-shi* and maintained at 18°C. Third instar larvae were incubated in a water bath at 32°C for 5 min and experiments were performed at 30°C with warmed equipment. Pinched larvae were moved to agar plates and maintained at 25°C.

To remove sensory neurons or hemocytes, *ppk-GAL4*; *GAL4* (Osborne et al., 1997) was crossed to *UAS-mCD8; GFP* was crossed to *UAS-dTrpA1* and maintained at 18°C. Third instar larvae were incubated at 29°C for two days to induce apoptosis and observed under a stereo fluorescence microscope (M205FA, Leica, Wetzlar, Germany) to check the decrease of GFP signal, or used for the assays.

To inhibit AP activity, *GAL4* (Osborne et al., 1997) was crossed to *UAS-mCD8; GFP* was crossed to *UAS-shi* and maintained at 18°C. Third instar larvae were incubated at 29°C for two days, the pinched larvae were moved to agar plates and maintained at 25°C.

**Microbial infection and pinching**

The following pathogens were used for infection: *E. coli* (IFO3830) and *S. saprophyticus* (GTC0205). For larval infection, overnight bacterial cultures were concentrated by centrifugation, the pellet was washed with 70% ethanol. At total of 20 larvae of each genotype were homogenized in 1/100 ml 1× PBS, and the larvae were then placed on a cold phosphate-buffered saline (PBS), and the larvae were then placed on a cold microscope slide. Pinched larvae were moved to agar plates and maintained at 25°C.

To monitor survival, 60 larvae of each genotype were incubated at 29°C after infection and the surviving larvae were every 2 h during transfer to fresh apple juice plates.

To assess the bacterial load in larvae, a colony-forming unit (CFU) assay was performed. Larvae were collected and their surfaces were sterilized with 70% ethanol. At total of 20 larvae of each genotype were homogenized in...
500 μl nutrient broth (NB) bacterial medium, serially diluted, and plated onto NB medium plates.

For pinching larvae, MilliQ water was poured into Drosophila vials and the water and larvae were moved to Petri dishes. The middle part of third instar larvae were gently (0.2-0.25 MPa, Prescale, Fujifilm, Tokyo, Japan) pinched by forceps (Dumont, 0108-5-PO) for about 1 s (Fig. S3C), and then the larvae were moved to sealed Petri dishes containing apple juice agar. Melanization spots and GFP signals in larvae after pinching were observed using a fluorescent stereo microscope.

Rearing the axenic fly line
To obtain germ-free larvae, embryos were washed with bleach as described in Broderick et al. (2014). Briefly, embryos were rinsed in 70% ethanol for 1 min, placed in a 2.5% solution of sodium hypochlorite for 2 min, and then washed with 70% ethanol for 2 min. Embryos were then rinsed in sterile MilliQ water. Embryos were transferred to sterile foods and developed to larvae.

To check the axenic state, bacterial DNA was extracted from whole larvae and assessed by real-time qPCR using 16S RNA primers (Suau et al., 1999).

Total RNA isolation, real-time qPCR, and microarray analysis
Larvae infected with bacteria or pinched by forceps were collected. Total RNA (1 μg) isolated from around 10 larvae using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used for cDNA synthesis with ReverTra Ace reverse transcriptase (Toyobo Ltd., Osaka, Japan) and oligo (dT) 15 primers (Promega, Madison, WI, USA). Using first-strand cDNA (0.5 μl), real-time qPCR was performed using a LightCycler (Roche Diagnostics, Roswell, GA, USA). rpL32 was used as the internal control. The following primers were used for real-time qPCR (F=forward, R=reverse): rpL32: 5′-AGA TCG TGA AGA AGC GCA CCA AG-3′ (F), 5′-CAC CAG GAA CTT GTA GCC CTTC CTT GGT G-3′ (R); Dsc: 5′-TTG TCT GCC CTC TTC GCT GTG CTG-3′ (F), 5′-GCA TCC TTT GCA CCA GCC TCT CA-3′ (R); Dps: 5′-GGT CAT CAT TGC TGT CCG GGT AC-3′ (F), 5′-CCA AGT GTC GCT CAT ATC TCC C-3′ (R); 125 RNA: 5′-TGG CGG TAT TTT AGT CTA TCT AGA GG-3′ (F), 5′-TAA GCT ACA CCT TGA TCT GA-3′ (R); and 16S RNA: 5′-CAG GAT TAG ATA CGG TGG TAT C3′ (F), 5′-TAA CCA CAT GCT CCG CCTT-3′ (R).

For microarray analysis, total RNA from Drosophila larvae homogenized in TRIzol was isolated using an RNeasy kit (Qiagen, Venlo, The Netherlands). The RNA quality was checked using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Total RNA (1 μg) was amplified and labeled as complementary RNA (cRNA) using an IVT Labeling Kit (Affymetrix, Santa Clara, CA, USA). Affymetrix Drosophila Genome 2.0 arrays were hybridized with 30 μg labeled cRNA, washed, stained and scanned (Goto et al., 2010). Data were analyzed by R software (https://www.r-project.org/).

Statistical analysis
Statistical analyses were performed using the Student’s t-test or log-rank test, and P<0.05 was considered significant.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
H.K., A.H., and T.K. conceived this study; H.K., A.H., and T.K. designed the experiments; A.H. and T.K. found that pinching larvae with forceps induced Drosomycin expression; A.H. and T.K. performed the experiments in Figs 1 and 2; H.K. performed the experiments in Figs 2-4; H.K., A.H., and T.K. analyzed the data; T.K. wrote the draft; H.K. and A.H. prepared the figures; all authors finalized the manuscript; T.K. led the entire project, and S.K. oversaw the study.

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Data availability
Microarray data have been deposited in GEO under accession number GSE94668 (available at: www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94668).

Supplementary information
Supplementary information available online at http://dmm.biologists.orglookup doi/10.1242/dmm.027102 supplemental

References


