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Genetic evidence of a redox-dependent systemic wound response via Hayan Protease-Phenoloxidase system in *Drosophila*

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Systemic wound response (SWR) through intertissue communication in response to local wounds is an essential biological phenomenon that occurs in all multicellular organisms from plants to animals. However, our understanding of SWR has been greatly hampered by the complexity of wound signalling communication operating within the context of an entire organism. Here, we show genetic evidence of a redox-dependent SWR from the wound site to remote tissues by identifying critical genetic determinants of SWR. Local wounds in the integument rapidly induce activation of a novel circulating haemolymph serine protease, Hayan, which in turn converts pro-phenoloxidase (PPO) to phenoloxidase (PO), an active form of melanin-forming enzyme. The Haemolymph Hayan-PO cascade is required for redox-dependent activation of the c-Jun N-terminal kinase (JNK)-dependent cytoprotective program in neuronal tissues, thereby achieving organism level of homeostasis to resist local physical trauma. These results imply that the PO-activating enzyme cascade, which is a prominent defense system in humoral innate immunity, also mediates redox-dependent SWR, providing a novel link between wound response and the nervous system.

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Introduction

In any multicellular organism, local disruption of the body integument by physical forces from external sides is readily sensed and subsequently leads to systemic wound response (SWR) to resist physical trauma (Foex, 1999; Delessert *et al*, 2004; Schilmiller and Howe, 2005; Lenz *et al*, 2007; Lowry and Calvano, 2008). In arthropods, one of the most visible wound responses is activation of circulating phenoloxidase (PO) in the blood-like haemolymphs, which is responsible for the melanin formation that primarily occurs around the wound site (Ashida and Yamazaki, 1990; Kanost *et al*, 2004; Cerenius *et al*, 2008). More than a century ago, Pasteur (1870) noted melanization around the wounded area in the integument of silkworm larvae during his study of silkworm diseases. PO, a key enzyme involved in melanin formation, catalyses the oxygenation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to the corresponding *o*-quinones. Melanins are heteropolymers derived from *o*-quinone (Ashida and Yamazaki, 1990; Kanost *et al*, 2004; Cerenius *et al*, 2008). Due to the propensity of quinone for redox cycling, melanin precursors are potential sources of reactive oxygen species (ROS; Nappi and Vass, 1993; Slepneva *et al*, 1999; Komarov *et al*, 2005). Biochemical studies have shown that PO is normally present in its enzymatically inactive precursor form, Pro-PO (PPO) (Ashida and Ohnishi, 1967). Conversion PPO to PO is achieved by limited proteolysis through a PO-activating cascade that includes sequential activation of multiple serine proteases (Ashida and Yamazaki, 1990; Kanost *et al*, 2004; Cerenius *et al*, 2008; Bidla *et al*, 2009). Although the exact mechanism governing PO activation in *Drosophila* has yet to be elucidated, PO-activating cascades can be initiated in response to infection or wound (Ashida and Yamazaki, 1990; Kanost *et al*, 2004; Cerenius *et al*, 2008; Zou *et al*, 2010). PO activity contributes to scab formation at the epithelial wound site, which is believed to be important to provide structural stability (Galko and Krasnow, 2004). Furthermore, by-products produced during melanin biosynthesis are cytotoxic; therefore, PO activity is also believed to contribute antimicrobial action (Zhao *et al*, 2007). Indeed, the involvement of PO-activating cascade in host immune response to diverse pathogen infections has been illustrated in *Drosophila* (Ayres and Schneider, 2008). However, the exact *in vivo* role of PO in response to wounding has yet to be elucidated.

Another response that occurs at the site of epithelial wounds is the induction of dopa decarboxylase and tyrosine hydroxylase (Mace *et al*, 2005). These enzymes are known to provide the substrates of PO for formation of the melanin and the cuticular skeleton in epithelial cells. Extracellular signal-regulated kinase (ERK) activation is necessary for the activation of *grainy head* gene product, which in turn acts as a transcription factor to induce *dopa decarboxylase* expression at the wound site (Mace *et al*, 2005). Furthermore, genetic studies have shown that c-Jun N-terminal kinase (JNK) activation in wounded epithelial cells is required for

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wound-healing processes such as epidermal cell spreading and re-epithelialization (Ramet *et al*, 2002; Galko and Krasnow, 2004; Campos *et al*, 2010; Lesch *et al*, 2010). In addition to these findings, genome-wide analysis has shown that various classes of wound-induced genes such as *growth arrest and DNA damage-inducible gene 45* were rapidly upregulated following sterile laser wounding (Stramer *et al*, 2008). All of these studies indicate that complex signalling pathways and subsequent wound-induced gene expression are required for integrated wound-healing processes.

Despite the aforementioned progress in elucidation of wound-induced cellular response during the wound-healing process, we still do not understand how the entire body mounts a program of SWR to resist local wounds. SWR is an essential biological program that requires coordinated communication between different tissues and ensures efficient host protection against physical trauma. Due to these complexities of intertissue communication between the wound site and remote tissues, the integrated signalling mechanism governing SWR within a living organism remains poorly understood.

In the present study, by taking advantage of genetic tools available in *Drosophila* system, we uncovered genetic evidence of a redox-dependent signalling pathway governing SWR for host protection against physical wounds. Integumental wound signals rapidly activate PO through a novel serine protease, Hayan. Hayan-dependent PO activation in haemolymph leads to the activation of a JNK-dependent cytoprotective program in neuronal tissues in a redox-dependent manner, thereby protecting the host against physical trauma. Our genetic analysis of redox-modulated SWR provides novel insight into the molecular mechanism by which an organism sets up coordinated tissue-to-tissue communication to achieve host homeostasis in response to a local wound.

Results

PO activity is involved in a redox-modulated SWR for host protection

It is well known that physical wounds rapidly lead to ROS production in organisms ranging from plants to humans (Orozco-Cardenas *et al*, 2001; Roy *et al*, 2006; Niethammer *et al*, 2009; Moreira *et al*, 2010; Woo *et al*, 2010). Given that ROS can diffuse across cells and act as second messengers to modulate diverse signalling pathways (Rhee, 2006), we investigated whether ROS are required for host SWR. To test this possibility, we used *Drosophila* adult flies carrying reduced ROS levels as a result of augmented antioxidant capacity through ubiquitous overexpression of the H_2O_2-removing antioxidant peroxiredoxin enzyme, jafrac1 (Radyuk *et al*, 2001; Rhee *et al*, 2005) or the immune-regulated catalase, IRC (Ha *et al*, 2005). Two different intensities of physical wounding, mild and strong, were used to damage the integument. This was achieved by using sterile needles of two different diameters (see Materials and methods). In the mild wounding condition, we found that transgenic animals overexpressing *jafrac1* or *IRC* showed ~15% mortality whereas control flies showed ~1% mortality (Figure 1A; Supplementary Figure S1A). Interestingly, the effect of antioxidant enzyme overexpression on wound-induced mortality was more pronounced when animals were subjected to strong wounding (i.e., ~50% mortality in transgenic animals overexpressing *jafrac1* or *IRC* versus ~15% mortality in control animals) (Figure 1A). To maximize the antioxidant effect on wound-induced host mortality, most of the experiments in this study were performed using the strong wounding condition unless otherwise stated. Consistent with the genetic modulation of antioxidant potential, animals pretreated with the antioxidant chemical (by ingesting *N*-acetylcysteine) also showed higher susceptibility to wounds compared with control animals (Supplementary Figure S2). These results showed that excess antioxidant potential was sufficient to render the host susceptible to physical wounds. However, both *Toll* pathway mutant and *IMD* pathway mutant flies showed normal survival rates against wounds (Figure 1A), indicating that Toll- and IMD-controlled innate immunity is dispensable for host resistance against physical wounds. Taken together, these results indicated that an appropriate level of ROS is somehow involved in host survival against wounds.

In arthropods, one of the most visible and rapid wound-induced responses is melanin formation at the wound site (Ashida, 1990; Kanost *et al*, 2004; Cerenius *et al*, 2008). The enzymatic activity of PO is responsible for the melanin formation. Given that melanin precursors are potential sources of ROS (Slepneva *et al*, 1999; Komarov *et al*, 2005), we investigated whether wound-induced PO activity is involved in SWR for host protection. To accomplish this, *Black cells* (*Bc*) mutants devoid of circulating haemolymph PO activity (Rizki *et al*, 1985) were subjected to physical wound. When the survival rate was examined, *Bc* mutants were found to be susceptible to wounds when compared with the control animals (Figure 1B). Time course analyses showed that similar wound-induced susceptibility was observed in both germ-free and conventionally reared *Bc* mutant animals (Figure 1C), confirming that lethality of the *Bc* mutant is not due to infection. Given that the molecular nature of *Bc* mutation is currently unknown and that *Bc* mutant exhibited various phenotypes that were unrelated to haemolymph PO deficiency, we attempted to rescue the wound-induced lethality of *Bc* mutants by introducing *PO* expression in the whole body. The results showed that overexpression of *PPO1* is sufficient to restore PO activity and host resistance against wounds (Figure 1B; Supplementary Figure S3). When we introduced *PO* expression in a tissue-specific manner (in the haemocytes of *Bc* mutant flies), ameliorated host survival rate following wounding was also observed (Figure 1B). Taken together, these results demonstrate that the wound-induced lethality of *Bc* animals can be rescued by increased PO activity.

The above results suggest that PO is involved in SWR for host protection. Given that an appropriate ROS level is likely needed for full host protection following wounding (Figure 1A) and that by-products of PO activity are ROS, we investigated whether the wound-induced mortality of *Bc* mutant animals is due to an insufficient ROS level. To test this possibility, we boosted host endogenous ROS levels by reducing host's antioxidant capacity. To accomplish this, we established a *Bc* mutant animal under a *jafrac1-RNAi* condition (Supplementary Figure S1B). Although enhancement of the endogenous ROS level by reducing *jafrac1* expression could not alter *Bc* phenotype as evidenced by black cell formation in the haemolymph (Supplementary Figure S4),

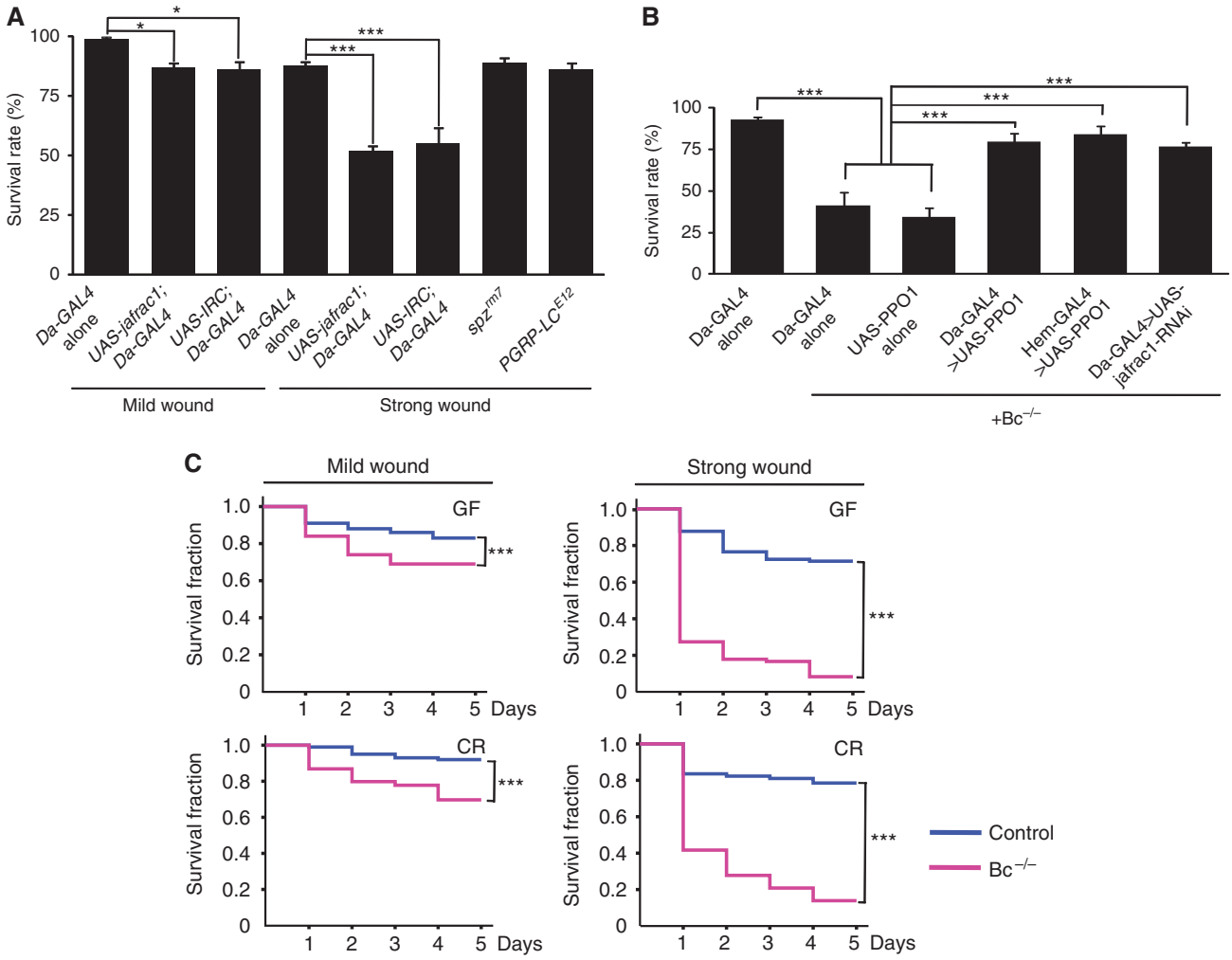


Figure 1 Phenoloxidase-mediated redox modulation is required for systemic wound response. In all experiments, survival in three or more independent cohorts comprising ~20 adults each was monitored following wounds. (**A**) Involvement of the redox system in wound response. To reduce endogenous ROS level, transgenic animals overexpressing antioxidant *jafrac1* enzyme (*UAS-jafrac1; Da-GAL4*) or *IRC* enzyme (*UAS-IRC; Da-GAL4*) were used. *Da-GAL4* alone was used as controls. Toll pathway mutant flies (spz^{rm7}) and IMD pathway mutant flies ($PGRP\text{-}LC^{E12}$) were also used. Data were analysed using ANOVA followed by Tukey's *post hoc* test, and values represent the mean ± s.e. ($^{*}P<0.05$, $^{***}P<0.001$) of at least three independent experiments. (**B**) Involvement of PO activity in redox-mediated wound response. The genotypes of the flies used in this study were as follows: Da-GAL4 alone (*Da-GAL4/+*); $Bc^{-/-}$ + Da-GAL4 alone (Bc^{1}; *Da-GAL4/+*); $Bc^{-/-}$ + UAS-PPO1 alone (Bc^{1}; *UAS-PPO1/+*); $Bc^{-/-}$ + Da-GAL4 > UAS-PPO1 (Bc^{1}; *UAS-PPO1/Da-GAL4*); $Bc^{-/-}$ + Hemese-GAL4 > UAS-PPO1 (Bc^{1}; *UAS-PPO1/Hemese-GAL4*); $Bc^{-/-}$ + Da-GAL4 > UAS-jafrac1-RNAi (Bc^{1}; *UAS-jafrac1-RNAi/Da-GAL4*). Data were analysed using ANOVA followed by Tukey's *post hoc* test, and values represent the mean ± s.e. ($^{***}P<0.001$) of at least three independent experiments. (**C**) Survival rate of *Bc* mutant animals following infliction of different wound strengths. Germ-free (GF) and conventionally reared (CR) animals were used. The genotypes of the flies used in this study were as follows: Control (*Da-GAL4*) and $Bc^{-/-}$ (Bc^{1}; *Da-GAL4*). Log rank analysis (Kaplan–Meier analysis) was used ($^{***}P<0.001$).

it was sufficient to ameliorate wound-induced survival rates of *Bc* animals lacking PO activity (Figure 1B). Furthermore, *Bc* animals exposed to a non-lethal concentration of pro-oxidant (by ingesting paraquat) showed enhanced survival rates following wounding (Supplementary Figure S5). Taken together, our results indicate that the wound-induced mortality of *Bc* mutant animals is likely due to insufficient host ROS levels, and that PO activity acts as an upstream event of redox-modulated SWR for host protection.

Screening of CLIP serine proteases involved in PO-mediated SWR

The CLIP-domain containing serine protease (CLIP serine protease) family plays an important role in many aspects of physiology including melanization and Toll activation (Ligoxygakis *et al*, 2002a; Jang *et al*, 2006, 2008; Kambris *et al*, 2006; El Chamy *et al*, 2008). Two CLIP serine proteases, MP1 (CG1102) and MP2/PAE1 (CG3066), are known to act as upstream enzymes for PO activation by converting PPO to PO in response to microbial infections (Leclerc *et al*, 2006; Tang *et al*, 2006; Ayres and Schneider, 2008). When we subjected RNAi-based knockdown (KD) animals of these proteases to physical wounds, the wound-induced survival rate of these animals is similar to that of control flies (Figure 2A). These results suggest that previously identified PO-activating enzymes such as MP1 and MP2/PAE1 are not involved in PO-mediated SWR under our experimental condition of physical wounding.

These findings also suggest that physical wounds require a novel CLIP serine protease for PO activation during SWR. A total of 24 serine proteases containing a single CLIP domain exist in the *Drosophila* genome (Ross *et al*, 2003; Jang *et al*, 2008). To investigate the possible involvement of a novel CLIP serine protease in the PO-mediated SWR for host protection, RNAi-based KD or mutant animals for each of 24 CLIP serine proteases were subjected to physical wound. When the wound-induced mortality of these animals was examined, the KD animal for *CG6361*, which we named the *Hayan* gene, showed the lowest survival rate (Figure 2B). These results indicate that the *Hayan* gene encoding previously uncharacterized CLIP serine protease is involved in SWR and is required for host protection against wounds.

Generation and phenotype analysis of fly line carrying the CG6361 null mutant allele, CG6361^{Hayan}

As both *Hayan* and *PO* are involved in SWR for host protection, we conducted in-depth analysis of the *Hayan*. To accomplish this, we generated three *Hayan* mutant alleles by mobilizing the P-element inserted in the 3′ untranslated region of the *Hayan* gene (Figure 3A). Genomic sequencing

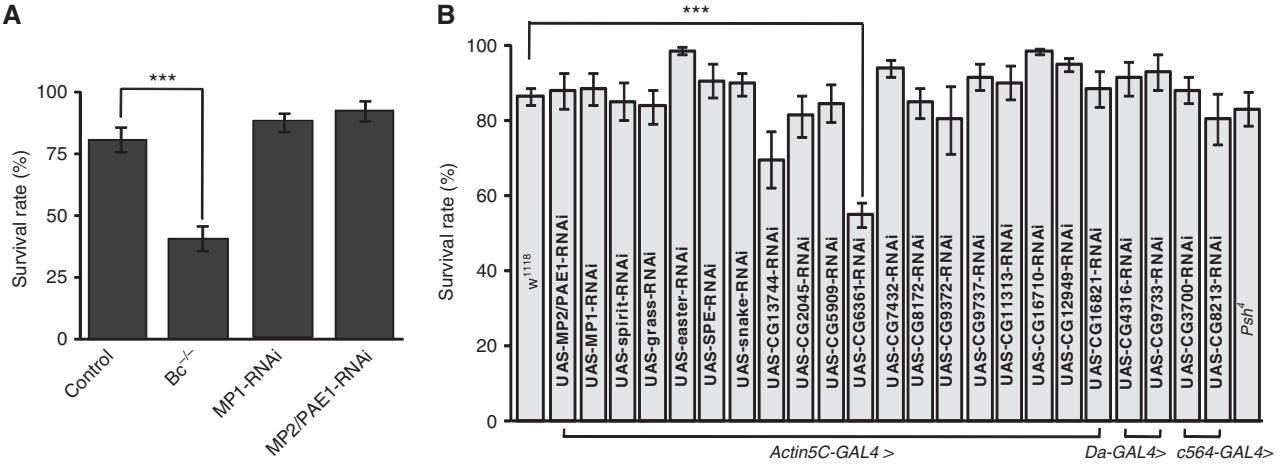
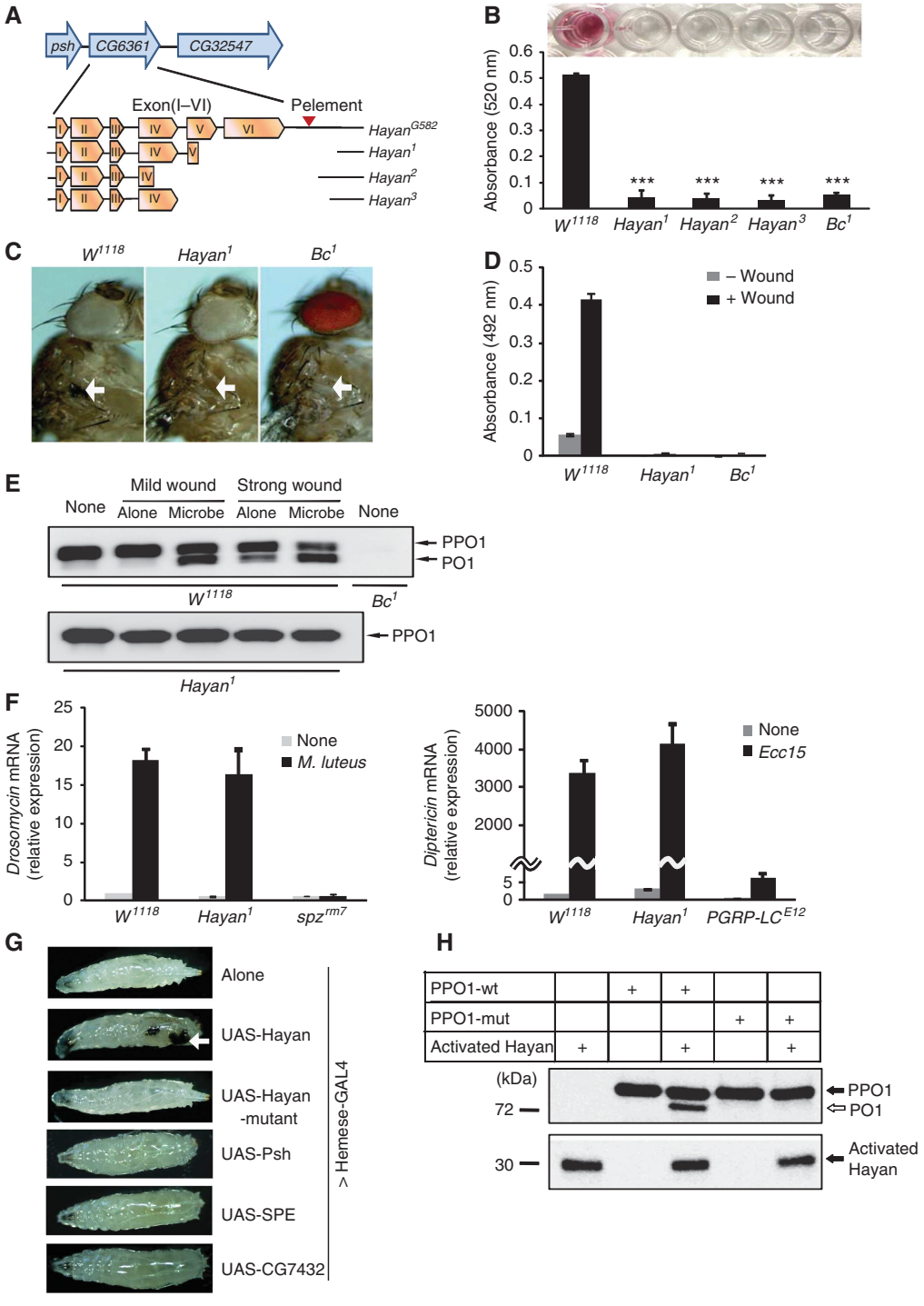


Figure 2 *CG6361* is involved in wound response. (**A**) Two known upstream proteases of PO activation, MP1 and MP2/PAE1, are not involved in wound response. The genotypes of the flies used in this study were as follows: Control (*Da-GAL4/+*); $Bc^{-/-}$ (*Bc^{1}*; *Da-GAL4/+*); MP1-RNAi (*UAS-MP1-RNAi/+; Da-GAL4/+*); MP2/PAE1-RNAi (*UAS-MP2-RNAi/+; Da-GAL4/+*). Data were analysed using ANOVA followed by Tukey's *post hoc* test, and values represent the mean ± s.e. (***$P<0.001$) of at least three independent experiments. (**B**) Identification of *CG6361* as a CLIP protease involved in wound response. Transgenic *Drosophila* strains that each contained an inducible *UAS-RNAi* construct against an independent CLIP protease gene were subjected to wound. *Actin5C-GAL4* or *Da-GAL4* driver was used to drive the expression of a hairpin RNA for all RNAi flies except for *CG3700-RNAi* and *CG8213-RNAi* flies, which were used with *c564-GAL4* due to the lethality of *Da-GAL4* in these strains. *psh^{4}* mutant flies were used for *Persephone* gene. Data were analysed using the Kruskal–Wallis test followed by the Mann–Whitney *U*-test using Bonferroni correction to adjust the probability. Values represent the mean ± s.e. (***$P<0.001$) of at least three independent experiments.

Figure 3 Characterization of a novel CLIP serine protease, *Hayan*. (**A**) Schematic representation of *Hayan* locus and neighbouring genes. *Hayan* mutant alleles were generated by using a P-element insertion line, *$Hayan^{G582}$*. (**B**) Spontaneous PO activity is abolished in *Hayan* mutants. Total extracts from five pupae were used to measure PO activity as described in Supplementary Materials and Methods. Data were analysed using ANOVA followed by Tukey's *post hoc* test, and values represent the mean ± s.e. (***$P<0.001$) of at least three independent experiments. (**C**) Wound-induced melanization is abolished in *Hayan* or *Bc* mutants. Wound areas are indicated by white arrows. (**D**) Wound-induced PO activity. Adult flies were subjected to wounding and the haemolymph from flies at 3 h post wounding was used to examine PO activity. (**E**) Both wound-induced and microbe-induced cleavage of PPO1 to PO1 are abolished in *Hayan* mutant. Germ-free animals were used in this experiment. Adult flies were subjected to different wound strengths (mild or strong) in the absence of microbes (wound alone) or in the presence of microbes (wound + microbe). In the case of septic wound, the flies were subjected to wound using a needle previously dipped into a concentrated pellet (OD = 200) of a mixture of Gram+ *Micrococcus luteus* (*M. luteus*) and Gram− *Erwinia carotovora carotovora 15* (*Ecc15*). Haemolymph from flies was obtained at 3 h post wounding to examine PPO1 cleavage. Western blot analysis was conducted using anti-PO1 antibody. (**F**) Toll and IMD pathways are intact in *Hayan* mutants. Flies were subjected to septic infection. Wild-type flies, Toll pathway mutant flies (*spz^{rm7}*) and IMD pathway mutant flies (*PGRP-LC^{E12}*) were also used as controls. Quantitative real-time PCR analysis of *Drosomycin* (at 24 h post infection) and *Diptericin* (at 6 h post infection) gene transcription was conducted following Gram+ *M. luteus* and Gram− *Ecc15* infection, respectively. The target gene expression of non-infected wild-type flies was arbitrarily set to 1, and the results are shown as relative expression levels. (**G**) Spontaneous melanization observed in *Hayan* transgenic animals. Haemocyte-specific *Hemese-GAL4* was used to induce *Hayan* overexpression. The genotypes of the flies used in this study were as follows: *Hemese-GAL4* alone (*Hemese-GAL4/+*); *Hemese-GAL4>UAS-Hayan* (*UAS-Hayan/+; Hemese-GAL4/+*); *Hemese-GAL4>UAS-Hayan*-mutant (*UAS-Hayan-mutant/+; Hemese-GAL4/+*); *Hemese-GAL4>UAS-Psh* (*UAS-Psh/+; Hemese-GAL4/+*); *Hemese-GAL4>UAS-SPE* (*UAS-SPE/+; Hemese-GAL4/+*); *Hemese-GAL4>UAS-CG7432* (*UAS-CG7432/+; Hemese-GAL4/+*). (**H**) The activated form of Hayan can cleave PPO1 to PO1 *in vitro*. C-terminal epitope-tagged recombinant proteins (HA epitope-tagged activated form of Hayan (Activated-Hayan), V5 epitope-tagged wild-type form of PPO1 (PPO1-wt) and V5 epitope-tagged cleavage mutant form of PPO1 (PPO1-mut)) were expressed in *Drosophila* l(2)mbn cells and then purified. Co-incubation of the activated-Hayan with PPO1-wt resulted in PO1. PPO1-mut with an altered cleavage site was not cleaved under these conditions. The same blot was incubated with anti-HA and anti-V5 antibody to detect Hayan and PO1, respectively. Figure source data can be found in Supplementary data.

analysis revealed that each of the mutant alleles lacked different regions containing exons 4, 5 and 6 (Figure 3A). Additionally, RT–PCR analysis showed that the full length of the *Hayan* transcript was absent from all three *Hayan* mutant flies (Supplementary Figure S6). Consistent to previous genome-wide mircoarray analysis (De Gregorio *et al*, 2002b), we found that *Hayan* transcripts are modestly upregulated in response to septic infection (~4-fold) in a Toll- and IMD-dependent manner (Supplementary Figure S7). Furthermore, we found that a sterile wound could also very modestly induce *Hayan* expression (~2-fold) and this modest wound-induced *Hayan* expression is also abolished in the absence of the Toll and IMD pathways (Supplementary Figure S7). To determine if Hayan is involved in the PO activation, we measured the spontaneous PO activity in the pupae extract. The results showed that spontaneous PO activity was completely abolished in all *Hayan* mutants (Figure 3B). Since these mutants showed similar phenotypes, we present the data obtained from $Hayan^1$ in this report. *Hayan* mutant animals apparently showed normal physiological phenotypes in terms of developmental rate and fertility. However, the *Hayan* mutant showed severely impaired melanization at the



wound site as in the case of *Bc* mutants (Figure 3C). Consistent with this observation, wound-induced PO activity in the adult haemolymph was also abolished in the *Hayan* mutant (Figure 3D). When we examined PPO1 cleavage to PO1 following aseptic and septic wounds, we found that wound alone could induce the formation of PO1, which could be further enhanced in the presence of microorganisms (i.e., septic wound; Figure 3E). This wound- and microbe-induced PPO1 cleavage to PO1 was completely abolished in the absence of *Hayan* (Figure 3E), indicating that *Hayan* is required for both wound- and microbe-induced PO activation. Because the *Hayan* mutant possesses intact antimicrobial peptide (AMP) transcript-inducing ability upon microbial infection (Figure 3F), Hayan is involved in the activation of PO, but not AMP induction. Consistent with these observations, we found spontaneous melanization in *Hayan* transgenic flies (Figure 3G). Neither overexpression of the proform of catalytically inactive Hayan nor overexpression of other CLIP serine proteases induced spontaneous melanization (Figure 3G), indicating that overproduction of Hayan zymogen is sufficient to force PO activation *in vivo*. To determine if Hayan can act directly on PPO1 to cleave into active form PO1 *in vitro*, we generated an activated form of Hayan consisting of only the catalytic domain after signal sequence cleavage (see Materials and methods). *In vitro* cleavage experiments showed that the recombinant activated form of Hayan can convert recombinant PPO1 to PO1 via limited proteolysis (Figure 3H). However, the activated form of Hayan was unable to cleave the mutant form of the PPO1 that had A^{52}-F^{53} instead of R^{52}-F^{53}, indicating that the cleavage site is situated between R^{52} and F^{53} (Figure 3H). Taken together, these results demonstrate that Hayan is involved in wound-induced PO activation and the subsequent melanization process, through the limited proteolysis of PPO1 to PO1.

Hayan completely suppresses melanization induced by serpin deficiencies

The serpin (Spn) family of serine protease inhibitors plays an important role in the negative regulation of PO-dependent melanization (Kanost, 1999). It has previously been demonstrated that Spn27A is involved in the negative regulation of the PPO-activating cascade via inhibition of the enzyme activity of the presently unidentified CLIP serine protease (De Gregorio *et al*, 2002a; Ligoxygakis *et al*, 2002b). Consequently, animals lacking Spn27A exhibited exaggerated melanization (De Gregorio *et al*, 2002a; Ligoxygakis *et al*, 2002b). Because Hayan appears to be required for PO activation, we tested whether the melanization observed in animals expressing *Spn27A-RNAi* could be reduced in the *Hayan* mutant genetic background. The results showed that spontaneous melanization found in *Spn27A-RNAi* animals could be completely abolished in the *Hayan* mutant background (Figure 4A). However, the activated form of Hayan did not form a complex with Spn27A (Supplementary Figure S8); therefore, Hayan may not be a direct target protease of Spn27A. Taken together, these results demonstrate that Hayan is involved in Spn27A-controlled PO activity.

Recently, Spn28D has also been identified as a negative regulator of melanization (Scherfer *et al*, 2008). Specifically, Spn28D has been shown to be involved in the control of haemolymph PO, particularly at the site of wounds (Scherfer *et al*, 2008). To investigate the possible relationship between Spn28D and Hayan, we generated double mutants for *Hayan* and *Spn28D*. The results showed that exaggerated melanization at the wound site observed in the *Spn28D* mutant animal was greatly abolished in the *Hayan; Spn28D* double mutant animals (Figure 4B). In addition to excess melanization at the wound site, spontaneous melanization was observed in the tracheae and spiracles of *Spn28D* larvae as well as the lateral sides of *Spn28D* pupae (Scherfer *et al*, 2008). These spontaneous melanizations were also completely abolished in *Hayan; Spn28D* double mutant larvae (Figure 4B). Furthermore, it is known that *Spn28D* mutation causes 100% lethality during the pupae stage (Scherfer *et al*, 2008). Interestingly, the lethality found in the *Spn28D* mutants was unable to be rescued by the *Bc* mutant background or in the absence of *MP*2 (Scherfer *et al*, 2008), indicating that this phenotype is independent of *Bc* and *MP2*. When we examined the *Hayan; Spn28D* double mutant animals, these organisms developed to the adult stage without any of the visible pupal lethality caused by *Spn28D* mutation (Figure 4C), indicating that *Hayan* mutation is capable of suppressing all phenotypes observed in the *Spn28D* mutant.

In addition to *Spn28D*, *Spn77Ba* was found to be involved in tracheal melanization (Tang *et al*, 2008). Tracheal melanization resulting from *Spn77Ba-RNAi* has been shown to induce systemic expression of the antifungal peptide *Drosomycin* in a melanization-dependent manner (Tang *et al*, 2008). To observe the genetic relationship between *Spn77Ba* and *Hayan*, we examined *Spn77Ba-RNAi*-induced tracheal melanization and *Drosomycin* expression in the absence of *Hayan*. The results showed that both tracheal melanization and *Drosomycin* expression were completely abolished in *Spn77Ba-RNAi* animals under a *Hayan* mutant genetic background (Figure 4D and E).

Taken together, these results demonstrate that Hayan protease is required for PO activation in different tissues controlled by three distinct Spn molecules. Because no available mutant or KD animals affecting PO activity, such as *Bc*, *MP1-RNAi* and *MP2/PAE1-RNAi* animals, were able to suppress all of the melanization phenotype induced by three different Spn molecules, Hayan is involved in diverse PO-activating cascades in *Drosophila*.

The Hayan is required for redox-modulated cytoprotective neuronal JNK activation and host protection against wounds

Given that *Hayan* is deeply involved in wound-induced PO activation, and that wound-induced PO activation is required for redox-modulated SWR, we first investigated whether *Hayan* is required for SWR for host survival. For this, we examined the survival rates of *Hayan* mutant following physical wounding. The result showed that physical wounds could lead to severe mortality in *Hayan* mutant flies (Figure 5A). A similar wound-induced mortality was also observed in germ-free animals, indicating that the mortality is not due to infection (Figure 5B). The physical wound in the presence of bacteria (i.e., septic infection) did not further reduce the host survival rate (Figure 5C) when compared with that of animals subjected to wound alone (Figure 5A and B), indicating that *Hayan* is mainly involved in SWR for host survival. The wound-induced mortality seen in *Hayan* mutants could be rescued by re-introducing the *Hayan* gene in haemocytes using the *hemese-GAL4* driver (Figure 6A;

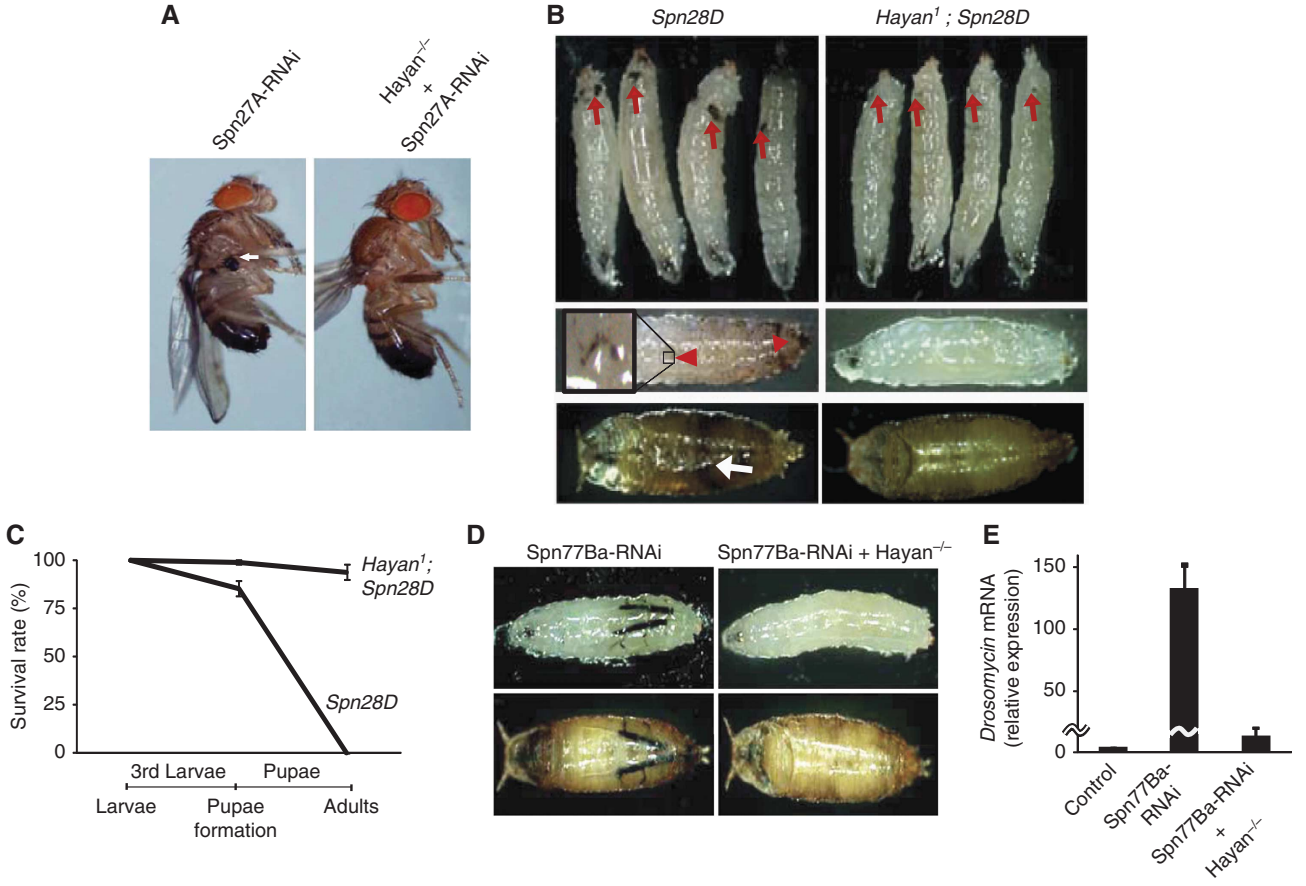


Figure 4 *Hayan* mutation completely suppresses melanization induced by serpin deficiencies. (**A**) Suppression of *Spn27A-RNAi*-induced melanization in the absence of *Hayan*. The white arrow indicates spontaneous melanization frequently observed in *Spn27A-RNAi* flies. The genotypes of the flies used in this study were as follows: Spn27A-RNAi (*UAS-Spn27A-RNAi, Da-GAL4/+*); Spn27A-RNAi + Hayan$^{-/-}$ (*Hayan*^{1}*; UAS-Spn27A-RNAi, Da-GAL4/+*). (**B**) Melanization of *Spn28D* mutant is abolished in the absence of *Hayan*. Exaggerated melanization at the wound sites (indicated by red arrows) of Spn28D larvae was greatly reduced in the absence of *Hayan* (upper panel). Melanization of the tracheae and spiracles (indicated by red arrowhead) in the Spn28D larvae (middle panel) and black dots (indicated by white arrows) at the lateral sides of the pupae (lower panel) were abolished in the absence of *Hayan*. (**C**) Lethality of *Spn28D* mutant is suppressed under a *Hayan* mutant genetic background. The survival rates of *Spn28D* mutants and *Hayan*^{1}*, Spn28D* double mutants were determined and were reported as the percentage of pupae formation and that of hatched adult flies from the larvae. (**D**) *Spn77Ba-RNAi*-induced tracheal melanization in the larvae (upper panel) and the pupae (lower panel) is abolished in the absence of *Hayan*. (**E**) *Drosomycin* induction seen in the *Spn77Ba-RNAi* is abolished in the absence of *Hayan*. The genotypes of the flies used in the study (**D**, **E**) were as follows: Spn77Ba-RNAi (*UAS-Spn77Ba-RNAi/+; Da-GAL4/+*); Spn77Ba-RNAi + Hayan$^{-/-}$ (*Hayan*^{1}*; UAS-Spn77Ba-RNAi/+; Da-GAL4/+*); control (*Da-GAL4/+*).

Supplementary Figure S9). These findings demonstrate that circulating haemolymph Hayan mediates SWR for host protection against wounds.

To examine the possible involvement of Hayan in redox-modulated SWR, we investigated whether the wound-induced mortality of *Hayan* mutant animals was due to an insufficient ROS level. To accomplish this, we boosted host endogenous ROS levels by establishing a *Hayan* mutant animal under a *jafrac1-RNAi* or *IRC-RNAi* condition. The results showed that enhancement of the endogenous ROS level of *Hayan* mutants by reducing antioxidant enzyme expression was sufficient to improve host survival following physical wounding (Figure 6A). Furthermore, *Hayan* mutant animals exposed to a non-lethal concentration of paraquat showed enhanced survival rates following wounding (Supplementary Figure S10). These results indicated that, as in the case of *Bc* mutant animals (Figure 1B; Supplementary Figure S5), wound-induced mortality of *Hayan* mutants is likely due to insufficient amounts of wound-induced ROS. These findings demonstrate that the haemolymph Hayan-PO1 system is required for redox-modulated SWR for host protection following wounding.

We next investigated the molecular mechanism by which Hayan promotes host protection against wounds. Previously, the JNK signalling cascade is known to be triggered by epithelial wounds (Ramet *et al*, 2002; Galko and Krasnow, 2004; Campos *et al*, 2010; Lesch *et al*, 2010). Genetic studies of *Drosophila* have demonstrated that JNK activity, especially in neuronal cells, is required for host resistance against stress by initiating a cytoprotective gene expression program (Wang *et al*, 2003). Given that overexpression of the antioxidant enzyme in neuronal cells, but not in other tissues, is sufficient to render the host susceptible to wounds (Supplementary Figure S11), and that ROS are diffusible molecules capable of directly activating JNK signalling (Rhee *et al*, 2000; Rhee, 2006), we investigated whether neuronal JNK signalling is involved in the host SWR. When we reduced JNK activity in neuronal cells by overexpressing

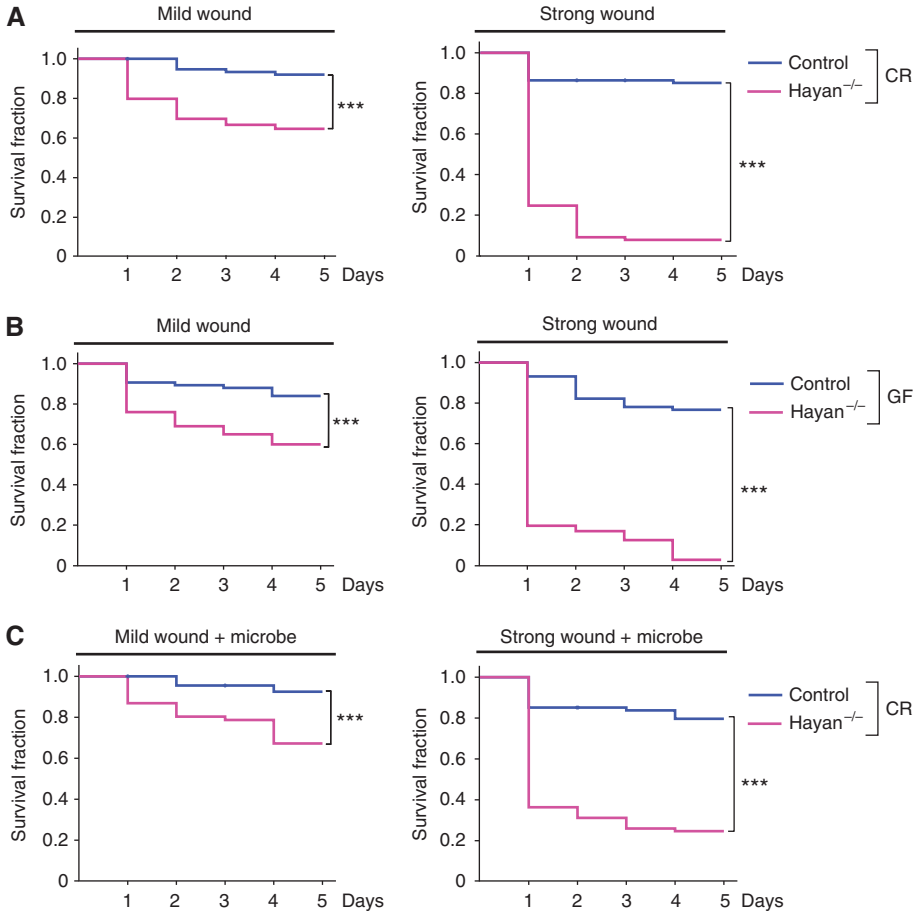


Figure 5 Survival rate of *Hayan* mutant animals following sterile wound or septic wound. Germ-free (GF) and conventionally reared (CR) animals were used. The genotypes of the flies used in this study were as follows: Control (w^{1118}) and $Hayan^{-/-}$($Hayan^{1}$). (**A**) Survival rate of CR flies following wound alone. (**B**) Survival rate of GF flies following wound alone. (**C**) Survival rate of CR flies following wound in the presence of bacteria. In this case, the flies were subjected to wound using a needle previously dipped into a concentrated pellet (OD = 200) of *Ecc15*. Log rank analysis (Kaplan–Meier analysis) was used (***$P<0.001$).

the dominant-negative form of JNK (JNK-DN), we found that reduction of the JNK signalling potential in these cells was sufficient to increase host susceptibility to physical wounds (Figure 6B). Control experiment showed that overexpression of JNK-DN in non-neuronal cells such as intestine cells gave no effect on host susceptibility to wounds (Supplementary Figure S12). These results indicate that redox modulation and JNK activity in neuronal cells are required for host protection against physical wounds.

We next investigated whether an integumental wound signal could induce neuronal JNK activation. Immunostaining using antibody against the active form of JNK revealed that wounding of the integument can induce JNK activation in neuronal cells (Figure 6C), indicating that the local wound signal was transmitted from injured epidermal cells to neuronal cells. Importantly, when we examined wound-induced JNK activation in *Hayan* mutant animals, we found that wound-induced JNK activation in neuronal cells was severely impaired (Figure 6C), indicating that local signal transmission from injured epidermal cells to neuronal cells is likely achieved through haemolymph Hayan. We next investigated whether wound-induced mortality of *Hayan* mutant animals is due to insufficient neuronal JNK activation. It is well known that *puckered* encodes a phosphatase that downregulates JNK activity (Martin-Blanco *et al*, 1998). Because a reduced *Puckered* gene dose in *Puckered* heterozygosity is known to enhance JNK activation (Wang *et al*, 2003), we enhanced JNK activation of the Hayan animals by establishing a *Hayan* mutant animal under a *Puckered* heterozygous background. When these animals were subjected to wounds, we found that wound-induced JNK activation became visible (Figure 6C). Consequently, wound-induced mortality of *Hayan* mutant animals was greatly ameliorated in *Puckered* heterozygote animals (Figure 6D). Furthermore, the high wound-induced mortality of *Hayan* mutant animals was also rescued by overexpressing *Puckered-RNAi* or the wild-type form of JNK in neuronal cells (Supplementary Figure S13). Control experiment showed that overexpression of *Puckered-RNAi* in non-neuronal cells such as haemocytes did not affect host survival following wounding (Supplementary Figure S13). Taken together, these results demonstrated that the neuronal JNK acts as a downstream event of haemolymph Hayan activation, which is required for host protection during SWR.

To examine the relationship between ROS level and JNK activation, we investigated whether the absence of wound-induced JNK activation in neuronal cells in *Hayan* mutant animals was due to the low level of ROS in neuronal cells that

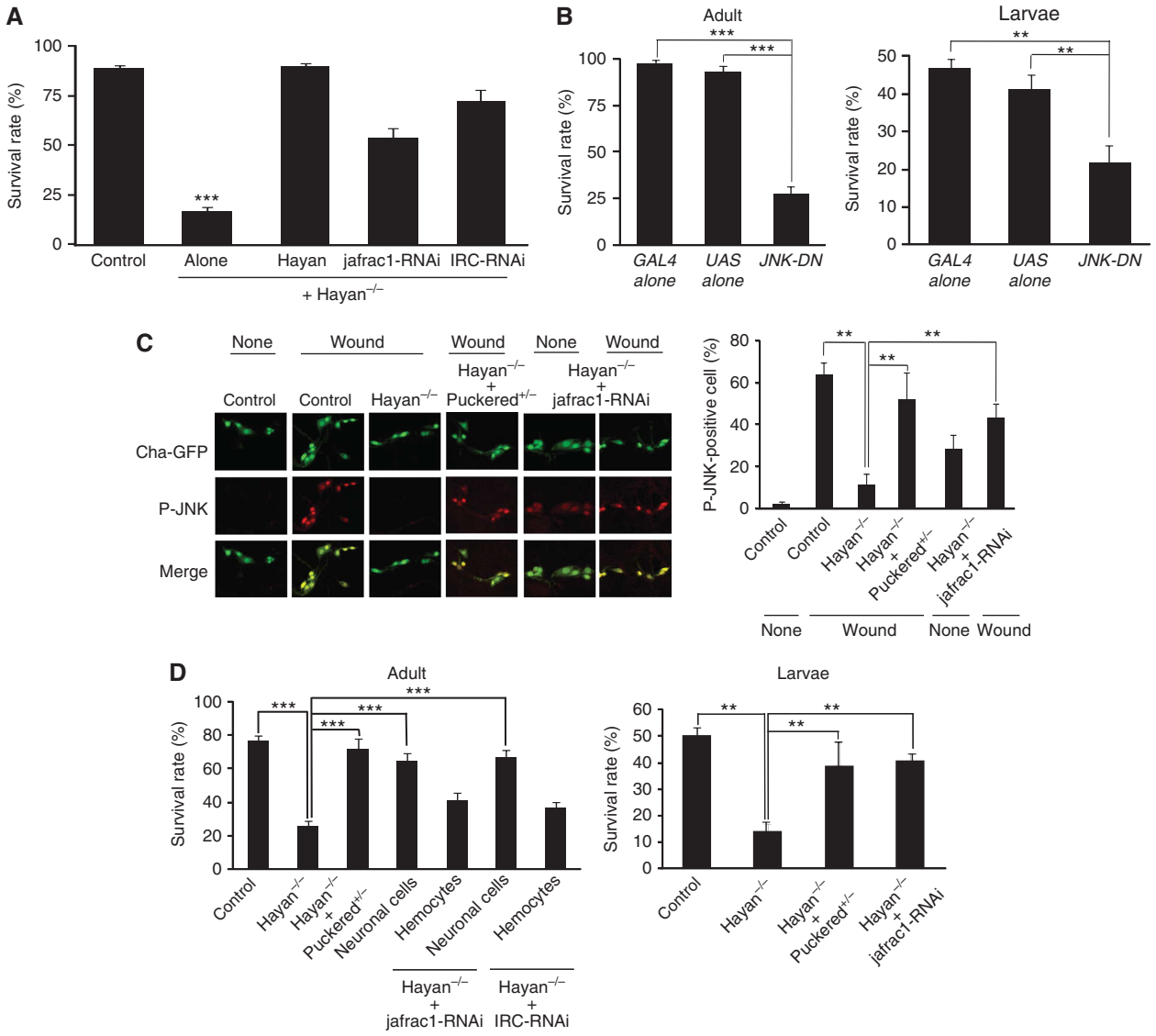


Figure 6 Hayan is required for redox-mediated neuronal JNK activation for host protection against wounds. In all experiments, survival in three or more independent cohorts comprising ~20 animals each was monitored following wounds. (**A**) Hayan is required for redox-mediated SWR for host protection. The genotypes of the flies used in this study were as follows: Control (w^{1118}); Hayan$^{-/-}$ (*Hayan*^{1}); Hayan$^{-/-}$ + Hayan (*Hayan*^{1}*; UAS-Hayan/+; Hemese-GAL4/+*); Hayan$^{-/-}$ + jafrac1-RNAi (*Hayan*^{1}*; UAS-jafrac1-RNAi/Da-GAL4*); Hayan$^{-/-}$ + IRC-RNAi (*Hayan*^{1}*; UAS-IRC-RNAi/+; Da-GAL4/+*). Data were analysed using ANOVA followed by Tukey's *post hoc* test, and values represent the mean ± s.e. (***$P<0.001$) of at least three independent experiments. (**B**) JNK activity in the neuronal cells is required for host protection following wounding. Both adults and larvae were used in this study. In the case of larvae, animals were subjected to pinch wounding as described in Materials and methods. To block the JNK activity, dominant-negative form of JNK (JNK-DN) was overexpressed in the neuronal cells. The genotypes of the flies used in this study were as follows: GAL4 alone (*Cha-GAL4/+*); UAS alone (*UAS-JNK-DN/+*); JNK-DN (*UAS-JNK-DN/+; Cha-GAL4/+*). Data in left panel were analysed using ANOVA followed by Tukey's *post hoc* test, and data in right panel were analysed using Kruskal–Wallis test followed by the Mann–Whitney *U*-test using Bonferroni correction to adjust the probability. Values represent the mean ± s.e. (**$P<0.01$, ***$P<0.001$) of at least three independent experiments. (**C**) The Hayan is required for wound-induced JNK activation in the neuron in a redox-dependent manner. The third instar larvae were subjected to wounds and the phosphorylated active form of JNK (P-JNK) was examined by immunostaining (at 1 h post wounding) as described in Materials and methods. The P-JNK index was determined by dividing the number of P-JNK-positive and GFP-positive cells by the total number of GFP-positive cells and multiplying by 100. Data were analysed using the Kruskal–Wallis test followed by the Mann–Whitney *U*-test using Bonferroni correction to adjust the probability. Values represent the mean ± s.e. (**$P<0.01$) of at least three independent experiments. P-JNK index (right panel) and representative images of P-JNK staining (left panel). The genotypes of the flies used in this study were as follows: Control (*Cha-GAL4, UAS-GFP/+*); Hayan$^{-/-}$ (*Hayan*^{1}*; Cha-GAL4, UAS-GFP/+*); Hayan$^{-/-}$ + Puckered$^{+/-}$ (*Hayan*^{1}*; Cha-GAL4, UAS-GFP/+; Puc*^{E69}*/+*); Hayan$^{-/-}$ + *jafrac1-RNAi* (neuronal cells) (*Hayan*^{1}*; Cha-GAL4, UAS-GFP/UAS-jafrac1-RNAi*). (**D**) Neuronal JNK activation through the Hayan-PO system is required for host protection against wounds. Both adults and larvae were used in this study. The genotypes of the flies used in this study were as described in (**C**). Hayan$^{-/-}$ flies overexpressing *IRC-RNAi* in the neuronal cells (*Hayan*^{1}*; Cha-GAL4/UAS-IRC-RNAi*) were also used in this experiment. Hayan$^{-/-}$ flies overexpressing *jafrac1-RNAi* and *IRC-RNAi* in the haemocytes (*Hayan*^{1}*; UAS-jafrac1-RNAi/Hemese-GAL4* and *Hayan*^{1}*; UAS-IRC-RNAi/+; Hemese-GAL4/+*, respectively) were used as controls. Data were analysed using ANOVA followed by Tukey's *post hoc* test, and values represent the mean ± s.e. (**$P<0.01$, ***$P<0.001$) of at least three independent experiments.

were diffused from haemolymph. To test this possibility, we attempted to increase the local ROS levels in neuronal cells by reducing the antioxidant potential in these cells in *Hayan* mutants. To accomplish this, we generated *Hayan* mutant overexpressing *jafrac1-RNAi* specifically in neuronal cells. When we examined the wound-induced JNK activation state in these animals, we found that both basal and wound-induced JNK activation of *Hayan* mutants could be greatly restored by reducing the neuronal antioxidant potential (Figure 6C). Consequently, overexpression of *jafrac1-RNAi* or *IRC-RNAi* in the neuronal cells, but not in haemocytes, could ameliorate wound-induced mortality in *Hayan* mutants (Figure 6D). Taken together, these genetic analyses demonstrate that sequential activation of the haemolymph Hayan-PO system ensures appropriate ROS levels in neuronal cells that are necessary for redox-dependent JNK activation, thereby achieving organism level of homeostasis to resist local physical trauma.

Discussion

In any multicellular organism, physical wounds in local sites must transmit an alarm signal to long distance remote tissues to mount a whole body level of protection. This SWR is an evolutionarily conserved phenomenon from plants to flies to humans (Foex, 1999; Delessert *et al*, 2004; Schilmiller and Howe, 2005; Lenz *et al*, 2007; Lowry and Calvano, 2008). Due to the intrinsic nature of cross-communication among spatially separated tissues, the biological effect of SWR is only visible at the organism level, which makes it difficult to analyse the molecular mechanism by which SWR is achieved. In this study, we used a genetic model of *Drosophila* to address the genetic determinants of SWR. Our genetic analyses demonstrated that sequential activation of haemolymph enzymes, Hayan serine protease and PO, relays local wound signals to remote neuronal cells in a redox-dependent manner to initiate the JNK-dependent cytoprotective program for a systemic host protection (Figure 7).

In arthropods, it has long been observed that physical wounds rapidly initiate spontaneous PO activation and subsequent melanization around wound sites (Ashida, 1990; Kanost *et al*, 2004; Cerenius *et al*, 2008). It has been shown that mutant animals lacking haemolymph PO activity such as *Bc* had enhanced mortality upon epithelial injury (Ramet *et al*, 2002). PO-dependent melanin formation is known to be required for scab formation at the site of wounds, which maintains a stable homeostatic barrier (Galko and Krasnow, 2004). However, the molecular mechanism by which PO is involved in the host wound response is still not clear. Our genetic study revealed a novel role of PO wherein it is involved in the sensing of local epithelial wound signals and subsequent transmission of this danger signal to the neuronal tissues in a redox-dependent manner. Screening of the 24 CLIP serine protease family revealed the involvement of a novel CLIP protease, Hayan, in the PO-mediated wound response. Given that rapid PO activation and melanin formation were almost completely abolished in *Hayan* mutants, and that these mutants were highly susceptible to wounds as in the case of *Bc* mutant animals, wound-induced PO activation occurred in a *Hayan*-dependent manner. As Hayan is involved in *Spn27a*- and *Spn28D*-controlled haemolymph PO activation as well as in *Spn28D*- and *Spn77Ba*-controlled

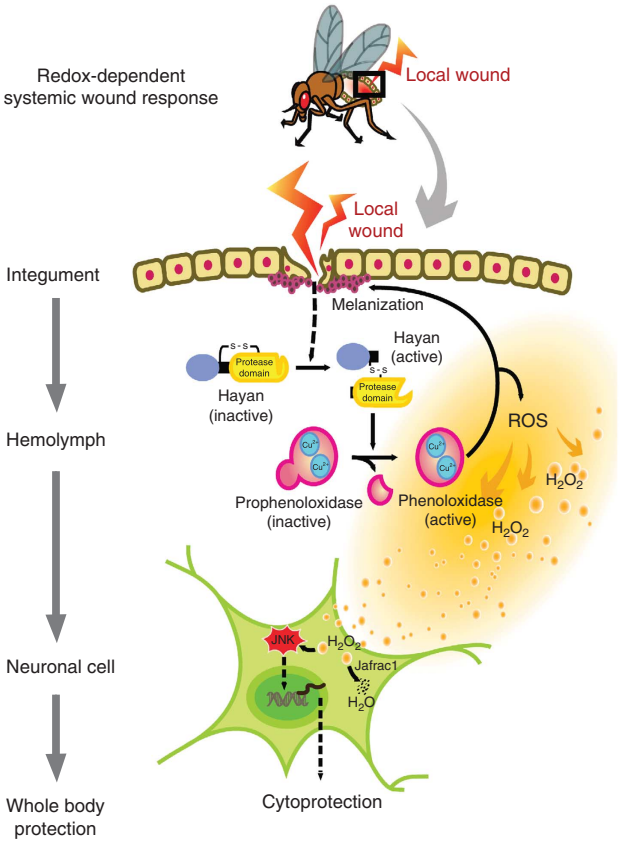


Figure 7 Model for redox-dependent systemic wound response. A novel Hayan serine protease in the haemolymph somehow senses integumental wounds to activate PO for the melanin formation. Hayan-dependent PO activation leads to ROS generation. ROS systemically diffuse through the haemocoel, acting as survival signals by activating the JNK-dependent cytoprotective program in neuronal tissues, thereby achieving host protection against physical trauma.

tracheal PO activation, Hayan is likely involved in PO activation in diverse tissues in response to distinct physiological conditions. Given that the Hayan-PO system is omni-present in haemolymphs and that the Hayan-PO system has a rapid activation rate, these characteristics may provide a spatio-temporarily ideal surveillance system for wound detection.

ROS are inevitable by-products of aerobic organisms that are often considered to be toxic molecules. However, it is generally accepted that hosts intentionally generate adequate amounts of ROS to modulate cellular signalling, which plays essential biological roles in response to specific physiological stimuli (Rhee *et al*, 2000; Rhee, 2006; Owusu-Ansah and Banerjee, 2009). During the wound-healing process, accumulation of ROS is observed locally at the wound sites in many organisms from plants to mammals (Orozco-Cardenas *et al*, 2001; Roy *et al*, 2006; Niethammer *et al*, 2009; Moreira *et al*, 2010; Woo *et al*, 2010). In these models, ROS act as second messengers that modulate many essential aspects of wound healing. For example, it has been shown that H_2O_2 modulates induction of defensive genes for wound repairs, migration of different cells to wound sites, and redox-dependent modulation of various growth factor signalling pathways (Orozco-Cardenas *et al*, 2001; Roy *et al*, 2006; Niethammer *et al*, 2009; Moreira *et al*, 2010; Woo *et al*, 2010). All of these studies

indicate that hosts employ ROS to modulate the signalling pathway at the local wound site. In addition to the role of ROS in the local wound area, the present study extends the role of ROS in the systemic response by showing that a local wound signal transduces into systemic signalling to protect the entire body from local injury. Overexpression of the H_2O_2-removing enzymes is sufficient to induce host mortality following wounding, indicating that an appropriate level of wound-induced ROS is required for host survival. Intracellular redox state is known to be controlled by the Nrf2, a member of 'cap-and-collar' family of transcription factor (Sykiotis and Bohmann, 2010; Hochmuth *et al*, 2011). Nrf2 is involved in the expression of antioxidant enzymes including peroxiredoxin (Motohashi *et al*, 2002; Hochmuth *et al*, 2011). Although reduction of *jafrac1* or *Nrf2* expression in neuronal cells is not sufficient to restore the wound-induced melanization in *Hayan* mutant flies (Supplementary Figure S14), it is sufficient to rescue the wound-induced mortality of these flies (Figure 6D; Supplementary Figure S15). These results clearly showed that the Hayan-PO system in haemolymph acts as an upstream event for wound-induced redox modulation in neuronal cells. It is conceivable that ROS generated via the Hayan- and PO-dependent melanin biosynthesis pathway that are freely diffusing into the neuronal cells act as intracellular second messengers capable of modulating JNK activation to protect the host against wounds. In mammals, ROS are known to promote TNF-α-induced JNK activation by inhibiting MAP kinase phosphatases (MKPs; Kamata *et al*, 2005). In this system, signal-dependent ROS cause oxidation and inhibition of JNK-inactivating phosphatases by converting their catalytic sulfhydryl group (−SH) of cystein to sulfenic (−SOH), resulting in sustained JNK activation. Given that ROS can readily diffuse in an open haemolymph system of insects, wound-induced ROS may directly activate JNK in neuronal cells, possibly inducing transient inactivation of *Drosophila* MKP(s) such as JNK-inactivating *puckered* or other upstream MKPs.

Our genetic study demonstrated that wound-induced ROS activate the JNK-dependent cytoprotective program, especially in neuronal cells. Previously, JNK activation at the edge of the epithelial wound was shown to be required for wound-healing processes such as epithelial cell movement and re-epithelialization (Ramet *et al*, 2002; Galko and Krasnow, 2004; Campos *et al*, 2010; Lesch *et al*, 2010). The results of the present study further demonstrated that JNK activation in neuronal cells is also required for efficient host protection against wounds, providing a novel role of JNK in the remote tissues of the wound site. We demonstrated that enhancement of JNK activation in neuronal cells by reducing *puckered* or *Jafrac1* expression is sufficient to rescue wound-induced mortality of flies lacking *Hayan*, demonstrating that redox-modulated JNK activation in neuronal cells acts as a downstream event of the Hayan-PO system during SWR. Because JNK activation in neuronal cells was shown to confer host protection to diverse stresses via the induction of subsets of cytoprotective genes (Wang *et al*, 2003), a similar JNK-induced cytoprotective program is likely operating in neuronal cells during wound response. Resistance and/or tolerance mechanisms of the hosts during different microbial infection have been well established (Ayres and Schneider, 2008; Schneider and Ayres, 2008). In this regard, it will be interesting to determine whether redox-modulated SWR is involved in resistance and/or tolerance mechanisms of the host in the case of physical trauma.

Recently, it has been shown that UV radiation activates the release of Eiger from epithelial cells that binds to its receptor Wengen on nociceptive sensory neurons underlying the epidermal sheet (Babcock *et al*, 2009), showing a link between wound epithelial site and nociceptive neuron via Eiger–Wengen signalling. It is presently unclear whether Eiger–Wengen signalling is required for host survival against a physical wound. Further genetic studies of Eiger–Wengen pathway in our model of SWR should help elucidating the possible relationship between Hayan-PO signalling and Eiger–Wengen signalling.

The role of PO in innate immunity has been well documented. PO plays an important role in antimicrobial and antiparasitic response in the haemolymph of arthropods (Ashida and Yamazaki, 1990; Kanost *et al*, 2004; Ayres and Schneider, 2008; Cerenius *et al*, 2008). Interestingly, the results of the present study demonstrated that a prominent enzyme in redox-mediated innate immunity, PO, is also involved in the signalling pathway of SWR, providing a novel link between wound response and the nervous system. As physical wounds are often accompanied by infection due to septic conditions of the external environment, it is tempting to speculate that overlapping functions of PO in innate immunity and wound response would provide a considerable advantage to the host during evolution. Although the full picture of the integrated signalling pathway of wound response and the innate immune pathway at the organism level is still incomplete, our findings regarding redox-modulated SWR provide novel insight into the evolutionarily conserved host response against physical wounds and emphasize the value of the *Drosophila* genetic model system for elucidation of a coordinated intertissue response to wounds.

Materials and methods

Wound experiment

All animals were maintained on standard cornmeal-agar medium at 25°C. Germ-free animals were generated as describe previously (Ryu *et al*, 2008). In all cases, survival in three or more independent cohorts comprising ∼20 animals each was monitored over time. Adult flies (7–8-day old) were subjected to wounding experiments. Two different intensities of physical wounding, mild and strong, were used. In mild wounding, the thorax of the animal was pricked (depth of wound is approximately equivalent of one fourth of thorax depth) using a sterile needle (diameter: ∼5 μm). In strong wounding, the thorax of the animal was completely penetrated using a sterile needle (diameter: ∼50 μm). Animals that died within 2 h post wounding were discarded. In the case of septic wound, the wound procedure was exactly the same as sterile wound except that the needle was previously dipped into a concentrated pellet of microbial culture before wounding. For the larvae, the third instar larvae were pinched with dissecting forceps at the posterior part of their body for 5 s. The survival rates were scored at 24 h post wounding in all experiments except some time course analysis of survival experiments.

Recombinant proteins

The pMT/V5-His vector (Invitrogen) was used to induce protein expression in *Drosophila* l(2)mbn cells. Wild-type PPO1 (PPO1-wt) is a C-terminal V5-His epitope-tagged full-length PPO1 (amino acids 1–690), while C-terminal V5-His epitope-tagged mutant form of PPO1 (PPO1-mut) has a mutant zymogen activation site ($A^{52}\downarrow F^{53}$ instead of $R^{52}\downarrow F^{53}$) created by site-directed mutagenesis. The C-terminal HA–His epitope-tagged activated form of Hayan consists of the signal peptide of the Easter fused directly to the Hayan

catalytic domain (amino acids 126–379). *Drosophila* l(2)mbn cells were maintained as described previously (Kim *et al*, 2000). Transfection of these cells was conducted according to a standard protocol using $CaPO_4$. Expression of PPO1-wt, PPO1-mut or the activated form of Hayan was induced in cells by the addition of $CuSO_4$ to the culture medium at a final concentration of 500 μM for 48 h before harvesting (Kim *et al*, 2000). Recombinant proteins were purified using Ni^+-NTA resin according to the manufacturer's instructions. Purified PPO1-wt, PPO1-mut and the activated form of Hayan were used for the *in vitro* PPO1 cleavage assay.

Antibody

Glutathione S-transferases (GST) fusion protein containing NH_2-terminal region of PO1 (amino acids 53–200) were produced using *E. coli*. The recombinant GST-PO1 proteins were purified to homogeneity using a Glutathione sepharose 4B column according to the manufacturer's instructions (GE Healthcare). The purified recombinant proteins were then injected subcutaneously into rabbits to generate polyclonal antibody using standard methods. The anti-PO1 antibody did not crossreact with other PO isoforms such as PO2 and PO3. Anti-V5 antibody, anti-HA antibody and anti-JNK phosphospecific antibody were purchased from Invitrogen, Roche and Cell Signalling, respectively.

Immunostaining

To visualize neuronal cells, we used an animal expressing GFP in cholinergic neurons (*Cha-GAL4, UAS-EGFP*). The third instar larvae were dissected in PBS and then fixed for 20 min with 4% paraformaldehyde. The samples were incubated with 0.2% Triton X-100 in Tris-buffered saline (TBS) for 5 min and then washed three times for 5 min each with 0.1% Triton X-100 in TBS. The samples were incubated with 0.1% Triton X-100 in TBS containing 5% BSA for 1 h and further incubated with the same buffer containing anti-JNK phosphospecific antibody (dilution 1:100; Cell Signalling) for 16 h at 4°C. The samples were then washed five times for 5 min each with 0.1% Triton X-100 in TBS, and secondary antibody (dilution 1:200; Alexa Fluor® 568 goat anti-rabbit IgG, Invitrogen) was then applied for 20 min. Finally, the samples were mounted using mounting buffer (Vectorshield, Vector Laboratories Inc.) and analysed by confocal microscopy (Zeiss).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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