Published in final edited form as:

Biochim Biophys Acta. 2010 November ; 1800(11): 1153–1163. doi:10.1016/j.bbagen.2010.06.010.

Peroxiredoxin 5 modulates immune response in *Drosophila*

Svetlana N. Radyuk^{a,*}, Katarzyna Michalak^a, Vladimir I. Klichko^a, Judith Benes^a, and William C. Orr^a

^aDepartment of Biological Sciences, Southern Methodist University, 6501 Airline Rd., Room 113, Dallas, TX 75275, USA

Abstract

Background—Peroxiredoxins are redox-sensing enzymes with multiple cellular functions. Previously, we reported on the potent antioxidant function of *Drosophila* peroxiredoxin 5 (dPrx5). Studies with mammalian and human cells suggest that peroxiredoxins can modulate immune-related signaling.

Methods—Survivorship studies and bacteriological analysis were used to determine resistance of flies to fungal and bacterial infections. RT-PCR and immunoblot analyses determined expression of dPrx5 and immunity factors in response to bacterial challenge. Double mutants for *dprx5* gene and genes comprising the Imd/Relish and dTak1/Basket branches of the immune signaling pathways were used in epistatic analysis.

Results—The *dprx5* mutant flies were more resistant to bacterial infection than controls, while flies overexpressing dPrx5 were more susceptible. The enhanced resistance to bacteria was accompanied by rapid induction of the Imd-dependent antimicrobial peptides, phosphorylation of the JNK kinase Basket and altered transcriptional profiling of the transient response genes, *puckered*, *ets21C* and *relish*, while the opposite effects were observed in flies over-expressing dPrx5. Epistatic analysis of double mutants, using attacin D and Puckered as read outs of activation of the Imd and JNK pathways, implicated dPrx5 function in the control of the dTak1-JNK arm of immune signaling.

Conclusions—Differential effects on fly survivorship suggested a trade-off between the antioxidant and immune functions of dPrx5. Molecular and epistatic analyses identified dPrx5 as a negative regulator in the dTak1-JNK arm of immune signaling.

General significance—Our findings suggest that peroxiredoxins play an important modulatory role in the *Drosophila* immune response.

Keywords

Peroxiredoxin; *Drosophila*; infection; immune; signaling

1. Introduction

Peroxiredoxins (Prx) comprise a family of antioxidant enzymes that also play an important role in signal transduction. Studies with mammalian and human cells suggest that

© 2010 Elsevier B.V. All rights reserved

*Corresponding author. snradyuk@smu.edu. Tel: +1-214-768-2892. Fax: +1-214-768-3955..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

peroxiredoxins can modulate immune-related signaling. Thus it was determined that both Prx 1 and Prx 2 can inhibit NF- κ B activation, while Prx1 can also inhibit c-Jun N-terminal kinase (JNK) signaling, and thereby mediate cellular responses to pro-inflammatory molecules [1–4]. In contrast, Prx 4 can activate both NF- κ B and c-Jun N-terminal kinase and induce inflammation [5]. Prx 1 also interacts with the macrophage migration inhibitory factor, an important cytokine in the regulation of host immune and inflammatory responses [6]. The human Prx 2 regulates TNF signaling pathways via mitogen-activated protein kinases subsequent to TNF- α stimulation [7] and its orthologue from *Schizosaccharomyces pombe* is required for the peroxide-induced activation of the p38/JNK homolog, Sty1, which mediates stress-response signaling [8]. Thus, there are several lines of evidence suggesting that peroxiredoxins are involved in regulating pro-inflammatory stress responses and innate immunity.

Innate immunity is an ancient mechanism of defense against invading microorganisms and is evolutionary conserved from invertebrates to humans. *Drosophila*, like other insects, lacks an adaptive immune system and depends solely on the innate immune system to combat pathogens. In response to infection, *Drosophila* initiates a massive production of numerous antimicrobial peptides (AMPs), comprising the humoral immune reaction. The synthesis of AMPs is induced by the NF- κ B-like transcription factors, Dorsal, Dif and Relish, which are activated via two distinct NF- κ B signaling pathways, Toll and Imd [9,10]. Regulation of the immunity effector genes is also influenced by the JNK and JAK/STAT signaling pathways, which can act in a competing or cooperative mode [11–15]. To prevent detrimental effects of prolonged and excessive immune response, flies have developed mechanisms to repress immune signaling. Recently, several factors involved in the negative control of the immune system have been identified, including Caspar, which suppresses nuclear translocation of Relish [16], and POSH, which is involved in the ubiquitination and proteosomal degradation of dTak1 [17]. Immune response signaling can also be turned-off via the JNK and JAK/STAT pathways, which appear to inhibit activation of the Relish-dependent genes [12,18].

Here, we report that the negative regulation of the *Drosophila* immune response can be mediated by a redox-sensing enzyme, peroxiredoxin 5 (dPrx5). Prx 5 subtype differs from other 2-Cys peroxiredoxins by the mode of resolution of reactive intermediates and, unlike other 'traditional' 2-cys peroxiredoxins, forms intramolecular S-S bonds. Previously, we reported that dPrx5 plays an important role in protecting flies from oxidative stress and apoptosis and promotes longevity in *Drosophila* [19]. The *dprx5* null mutant exhibited a dramatically decreased survivorship relative to controls upon exposure to H₂O₂ or paraquat and a significantly reduced mean life span under normal conditions [19]. In the present study, we demonstrate that the *dprx5* mutant has greater resistance to bacterial infection and provide evidence that dPrx5 acts as a negative regulator of the immune response by modulating JNK-mediated signaling.

2. Materials and Methods

2.1. Fly strains and procedures

The *y w* strain has been maintained in this laboratory for >15 years. The Actin 5C-GAL4, Da-GAL4 and Tub-GAL4 driver lines were supplied by Dr. Blanka Rogina (University of Connecticut Health Science Center). P{Switch1}106 driver (stock # 8151) was obtained from the Bloomington Stock Center. A strain containing a P-element insertion in the coding region of *ird5*^{EY02434} (stock # 19825), *rel*^{E20} mutant (stock # 9457) and the P{UAS-bsk.DN}2 transgenic line (stock # 6409) were obtained from the Bloomington Stock Center. We received the mutant strain *key*¹ (deficient for *Kenny*) from Dr. Neal Silverman, University of Massachusetts Medical School, and the mutant strain *dtak1* from Dr. Dirk Bohmann, University of Rochester Medical Center. A deficiency strain *Df(1)dreddD3* lacking *dredd*

caspase was obtained from Dr. John Abrams, UT Southwestern Medical Center. The *key¹*, *dtak1*, *rel^{E20}* and *Df(1)dreddD3* alleles are described elsewhere [20–23]. The *dprx5* mutant and the UAS-dPrx5 transgenic lines are described in [19]. To exclude background effects on survivorship and AMP expression levels, P-element mutants, UAS transgenes or GAL4 driver lines were backcrossed with *y w* for 6–8 generations to obtain genetically homogeneous stocks. *Df(1)dreddD3*, *key¹*, *rel^{E20}* and *dtak1* alleles were crossed to *y w* or *dprx5* flies using a combination of appropriate balancers. In these cases, the experimental double mutants with *dprx5* background were derived in the same way as corresponding controls. For experiments flies were collected within 1–2 days after hatching and reared on a standard sucrose-cornmeal medium at 25°C for 8–10 days. The induction of P Switch system was elicited by feeding flies regular food containing 0.1 mg/ml mifepristone for at least 48 hours.

2.2. Generation of the UAS-dPrx5^{RN} transgenic strains

To generate a construct producing redox-negative (RN) peroxiredoxin 5, a conserved Cys79, which is essential for peroxidase activity of the peroxiredoxin 5 subtype, was replaced with a serine residue. A mutagenesis was performed utilizing Splicing by Overlapping Extension PCR (SOE-PCR) using primers containing a single nucleotide mismatch, where a *tgc* triplet encoding for a cysteine was converted to a *tcc* triplet coding for a serine. Then the fragment of the UAS-dPrx5 construct, obtained by digestion with NarI endonuclease, was substituted with the mutated fragment. Transgenics were obtained by injection into germ cells utilizing the services of the TheBestGene company (www.thebestgene.com).

2.3. Infection experiments

Septic infection was caused by pricking adult flies with a needle dipped in a 24 h culture of bacteria. Natural infection was elicited by feeding flies with a bacterial suspension in a Luria broth containing 1 % sucrose. Natural infection with *B. bassiana* was obtained by shaking flies for 1–2 min with a sporulating fungal culture. The *B. subtilis* strain BGSC1A1, the strains of *M. luteus*, *S. marcescens* (ATCC#8100) and *P. aeruginosa* were a kind gift of Dr. Christine Buchanan, SMU. *Beauveria bassiana* and *Pectobacterium carotovorum* were obtained from the ATCC (#9453 and #17799) and the *E. coli* strain used was an ampicillin-resistant transformant of the laboratory DH5-a strain.

2.4. Immunoblotting

Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad). 5–10 µg of protein extracts were resolved by 10% SDS-PAGE followed by transfer to PVDF membrane (Millipore) and immunoblots were performed as described previously [24]. The dPrx5 protein was detected with antibodies raised against a recombinant dPrx5 protein. To control for loading, anti-actin antibodies (MP Biomedicals) were used. Phosphorylated JNK (Basket) was analyzed with anti-phospho-JNK antibodies (Cell Signaling Technology); anti-JNK antibodies were obtained from Santa Cruz Biotechnology.

2.5. RT-PCR

Forward and reverse primers specific for different genes are indicated in the Table I. Real-time quantitative PCR was performed with cDNA aliquots obtained as described previously [25] and pairs of gene-specific fluorescent primers. Primers for the housekeeping gene (Actin) and AMP genes were labeled differentially (ea JOE-labeled versus FAM-labeled). Consequently, fluorescent signals obtained during co-amplification of a target gene and a house-keeping gene were detected using different channels. Alternatively, PCR reactions were performed with SYBR Green (Molecular Probes) added to PCR reaction mixtures, and signals obtained for a target gene were standardized against signals obtained for a house-keeping gene (rp49 or actin) in parallel sets of reactions. Fluorescent primers for real-time PCR were designed using D-

LUXTM Designer (Invitrogen) and quantitative real-time PCR analysis was performed using Rotor-GeneTM RG-3000 (Corbett Research) and software.

2.6. Statistical analysis

All analyses were performed using Prism 4.0a for Macintosh or Microsoft EXCEL software.

3. Results

3.1. The *dprx5* mutant is more resistant to infection

To investigate resistance to various microbes, we used at least 10 da old flies, when the increased mortality rate in the *dprx5* mutant is stabilized and becomes similar to that of the *y w* control [19]. Another reason for using flies of this age is that 10 da old flies are fully developed, which minimizes potential effects of variations in the levels of hormones, such as juvenile hormone and ecdysone, which may tremendously affect immunocompetence [26].

As indicated in the Introduction, the innate immune response in *Drosophila* entails at least two distinct signaling pathways: Toll and Imd. It is well known that Gram-negative bacteria induce humoral immunity mainly via the Imd pathway, while Gram-positive bacteria and fungi primarily activate the Toll pathway (reviewed in [27]), although the activation of either pathway is not always strictly selective and the immune response is more complex [28]. For instance, some Gram-positive bacteria, such as *B. subtilis*, are also sensed through the Imd pathway [10] and vice versa, Gram-negative bacteria, such as *P. aeruginosa* activates both Toll and Imd pathways to combat the infection [29].

When flies were infected with *B. bassiana*, survivorship in both controls and mutant flies was almost equivalent reaching approximately 30% by day 10 after infection (Fig. 1 A). We also investigated survivorship of flies infected with Gram-positive bacteria *M. luteus*; however no significant mortality was determined with this pathogen, since ~ 90% of both control and mutant flies survived after 4 days post-infection (data not shown). Similarly, we were not able to discern differences in survivorship of flies that were naturally infected with *P. carotovorum*, as only minor mortality was observed (data not shown). However, we were able to determine differences in recovery from septic injury with *B. subtilis*, which is known to activate the Imd pathway [10]. Infection with this bacterium caused approximately 80% death of control after 72 h post-infection, while almost 60% of *dprx5* flies survived infection (Fig. 1 B). The *dprx5* mutant had also slightly enhanced resistance to natural infection with *P. aeruginosa* that was particularly evident during 24–72 hours post-infection (Fig. 1 C). Septic injury with *E. coli* caused only moderate mortality (less than 30%) within 12 hours followed by recovery in both control and mutant flies (data not shown). However, bacteriologic analysis revealed that the mutant flies were cleared from infection significantly faster relative to the controls (Fig. 1 E). The opposite effects on resistance to bacteria were observed in flies expressing dPrx5 from the UAS-dPrx5 transgene using global Act-GAL4 or Da-GAL4 drivers in flies with the *dprx5* null background. Expression of dPrx5 at high levels severely affected survivorship of flies infected with *P. aeruginosa*, where median survival time of experimental flies was about one third of that in driver and transgene controls (Fig. 1 D). Together, these data indicate that the *dprx5* mutant has enhanced resistance to bacterial infection compared to its isogenic control. In contrast, high levels of dPrx5 reduced survival of infected flies. The data also suggest that the immune response triggered via the Imd pathway may be a cause of the observed enhanced resistance to bacteria.

3.2. AMP levels

To determine whether the observed resistance to bacteria in the *dprx5* mutant relies, at least in part, upon activation of the specific immune pathway(s), we measured levels of the key

effectors of humoral immunity, the anti-microbial peptides (AMP). We used infection with *B. bassiana* to trigger the Toll pathway and injury with *E. coli* to trigger the Imd pathway and evaluated the levels of different AMPs.

The basal AMP levels in mutant and control flies were almost undetectable except drosomycin, which was expressed in flies under normal conditions at approximately the same levels in both control and mutant flies (not shown). Infection with *B. bassiana* caused elevation in the levels of the anti-fungal AMPs, drosomycin (50–60 fold induction) and metchnikowin (8–12 fold induction), while other AMPs were not induced (Fig. 2 A and not shown data). Both drosomycin (Drs) and metchnikowin (Mtk) are induced primarily via the Toll pathway; however, there were no differences in the response between control and mutant flies after 24 h post-infection (Fig. 2 A). Septic injury with *E. coli* caused up-regulation of several tested AMPs after 8 h post-infection, although the induction of attacins (Att) and to a lesser extent dipterizin A (Dipt) and cecropin C (CecC) was notably pronounced in the *dprx5* mutant relative to control. Furthermore, the differences in the induction of attacin D (AttD) and cecropin C (CecC) between the *dprx5* mutant and *y w* control were statistically significant (Fig. 2 A). It is well known that the induction of attacins, cecropins and dipterizins is associated primarily with the Imd/Relish pathway; moreover, the activation of AttD and DiptA is controlled only by Relish [30]. The effects on the levels of AMPs, normally up-regulated in the *dprx5* mutant (Fig. 2 B), were reverted by ubiquitous (by 2.5–6 fold) over-expression of UAS-dPrx5, resulting in ~ 2 fold decrease in the AMP levels. Taken together, the observed differences in the induction of AMPs suggest that dPrx5 may modulate the immune response mediated via the Imd pathway.

We also investigated whether the observed AMP induction is a general response to potentially elevated oxidative stress in the *dprx5* mutant or a response to proliferating bacteria. To test this, we injected flies with live and killed bacteria and compared AMP levels. No differences in AttD or Dipt levels were found between flies pricked with live or thermo-inactivated bacteria, while significant differences in the AttD or Dipt levels were found between samples isolated from flies injured with a sterile needle and flies pricked with bacterial suspensions. In the latter, the response was significantly stronger (Fig. 2 C). Strong induction of dipterizin or attacin was also not observed in flies treated with paraquat for the periods from 1 h to 8 h (data not shown). Finally, we did not find any differences in the levels of H₂O₂ in the whole body lysates or mitochondria prepared from mutant and control flies (data not shown). Altogether, the data suggest that the observed early response in the *dprx5* mutant is not due to bacterial proliferation or elevated ROS levels but rather due to specific effects of dPrx5 on factors involved in regulating immunity.

3.3. Epistatic analysis

To position dPrx5 regulatory effects within specific immune pathway(s), we generated a series of double-mutant lines involving *dprx5* in combination with alleles that are known to reside in the pathways regulating *Drosophila* immunity. Since the induction of AMPs suggested that dPrx5 might modulate the Imd-dependent immune response, a phenotypic analysis was performed using flies that are mutant for genes of the Imd pathway. The immune response, triggered via the Imd pathway, is initiated after stimulation of pattern recognition receptors [31,32] and requires a receptor proximal complex, which includes Imd and dFADD [33,34]. The signal is subsequently transmitted through activation of the kinase complex DmIKK [35], by means of the kinase kinase dTak1 [21]. Activation of DmIKK is necessary for phosphorylation of the NF- κ B protein Relish, which is further processed by Dredd caspase [36]. The liberated RHD domain of Relish is then transported into the nucleus where it activates transcription of the AMP effector genes. We generated a series of double-mutant lines involving *kenny*, *ird5*, *dtak1*, *rel* or *dredd* alleles in combination with *dprx5*. It has been

previously reported that AMPs, triggered primarily by the Imd/Relish module, have significantly reduced levels in *kenny*, *dtak1*, *rel* or *dredd* mutants [22,35]. Indeed, we found that the levels of AttD, which was used as a read out gene for the Imd pathway, were almost undetectable in *dredd*, *rel* and *kenny* or *ird5* ten days old mutants infected with *E. coli* and were significantly reduced in the *dtak1* mutant (Fig. 3). The reduction in AttD levels was partially compensated by the absence of dPrx5 expression in the *dprx5;dtak1* double mutant, but not in the double mutants for dPrx5 and the IKK complex (*kenny* and *ird5*) nor the *dredd;dprx5* and *rel;dprx5* double mutants (Fig. 3).

The absence of compensatory effects in the IKK-Relish module suggests that the activity of Relish is still required for the induction of Imd-dependent AMPs in either normal or *dprx5* background. However, partial up-regulation of the Imd-dependent antimicrobial peptides, which occurs in the absence of dTak1 activity in the *dprx5* null background, suggested the presence of an alternate route for activation.

3.4. dPrx5 modulates the JNK pathway

We examined whether dPrx5 may affect AMP expression via the Imd/JNK branch of the immune signaling pathways. It has been shown that Imd-dependent activation of *Drosophila* innate immunity bifurcates downstream of dTak1, leading to the activation of both NF- κ B and JNK pathways [11,13,37]. We evaluated activation of the JNK pathway in the *dprx5* mutant background by assessing phosphorylation patterns of JNK kinase (Basket) following infection. Activation of Basket occurred rapidly in the *yw* control and was sustained for at least 1 h after infection before dropping off. In the *dprx5* mutant, activated Basket was already observed prior to infection, showed no further activation in response to infection, and, in fact, declined significantly by 2 h post-infection (Fig. 4). In contrast, phosphorylation of Basket in flies over-expressing dPrx5 (UAS-dPrx5/Act) had a temporal profile, similar to that in the control but less pronounced (Fig. 4). Unlike phosphorylation, the expression levels of Basket (JNK) did not change in response to infection (data not shown).

The altered phosphorylation of Basket in flies under- or over-expressing dPrx5 resulted in compromised induction of transient response genes *puckered*, *ets21C* and *relish*, which was evaluated by measuring their mRNA levels. As determined by RT-PCR analysis, induction of the gene coding for the dual specificity phosphatase Puckered, a target gene of the JNK pathway, was observed in both the *dprx5* mutant and control flies, although the response was more robust in the *dprx5* mutant and less pronounced in flies over-expressing dPrx5 (Fig. 5 A). We also determined changes in the response of another putative target of JNK signaling, transcription factor Ets21C [15,38]. As noted in Fig. 5 B, induction of Ets21C was found to occur earlier in the *dprx5* mutant and was dampened in the dPrx5 over-expressor (Fig. 5 B). Similarly, the transcription factor Relish was found to exhibit a much more robust response in the *dprx5* mutant while that of the overexpressor was reduced (Fig. 5 C).

We also investigated whether peroxiredoxin 5 effects on the JNK pathway are due to its peroxidatic activity. To test this, we generated a UAS construct carrying a redox-negative Prx5 variant (UAS-dPrx5^{RN}), where catalytic cysteine-79 was replaced with redox-inactive serine-79 (see Materials and Methods). This type of amino acid substitution compromises only peroxidase activity of peroxiredoxin 5, and not other properties of this protein [39].

The catalytically inactive UAS-dPrx5^{RN} variant and normal UAS-dPrx5 were expressed with the Da-GAL4 driver in flies having a *dprx5* null background. For these experiments, transgenic lines expressing comparable levels of dPrx5 and dPrx5^{RN} proteins were selected (Fig. 6 C). Unlike UAS-dPrx5, which rescued the *dprx5* mutant phenotype from increased susceptibility to H₂O₂ or paraquat [19], redox negative UAS-dPrx5^{RN} failed to protect flies from oxidants (data not shown), thus confirming that the mutated form of peroxiredoxin 5 lost its antioxidant

activity. At the same time, the effects on the activation of the JNK pathway, observed in flies expressing normal dPrx5, were absent in flies expressing the redox-negative peroxiredoxin 5, whose phenotype was largely similar to that of the *dprx5* mutant. For instance, flies expressing dPrx5^{RN} exhibited induction of the downstream response genes Puckered and Ets21C, effects that were dampened in flies expressing wild type dPrx5 (Fig. 6 A and B). Similarly, the phosphorylated Basket, observed in the *dprx5* mutant prior to infection, was also observed in flies expressing the redox-negative peroxiredoxin 5 (Fig. 6 C). In contrast, kinase Basket was not phosphorylated in the absence of infection in *yw* flies and flies expressing normal dPrx5 (Fig. 6 C). Taken together, these data show that peroxiredoxin 5 modulates the JNK pathway by virtue of its peroxidase activity.

3.5. Expression of Imd- and JNK target genes is affected by dominant-negative Basket

To further test involvement of the JNK pathway in the induction of Imd-dependent AMPs, we generated flies with reduced JNK activity by expressing the dominant-negative form of Basket (Bsk^{DN}), which is nonactivatable due to a mutation in the phosphorylation site T181A [40]. Bsk^{DN}-expressing flies were generated in normal (*y w*) or *dprx5* null background using the mifepristone-inducible P{Switch1}106 driver that targets responder gene expression to the *Drosophila* fat body. Flies were fed regular food containing mifepristone for two days prior to infection, resulting in a 5–7 fold increase in the levels of Bsk^{DN} compared those of the endogenous Basket (Fig. 7 A). As anticipated, activation of the JNK pathway was significantly dampened in flies over-expressing the dominant-negative Basket protein, as determined by measuring mRNA levels of Puckered, a target gene of the JNK pathway (Fig. 7 B). While the induction of Puc was completely abrogated in the Bsk^{DN}-expressing flies, the effects on the induction of Ets21C were less pronounced but still significant (Fig. 7 C), which confirmed that Ets21C is at least partially regulated via the JNK pathway. The induction of Puckered or Ets21C was not restored in the absence of dPrx5, which suggests that dPrx5 acts upstream of Basket. To investigate the effects of the dPrx5 mutation in flies with normal and Basket-DN backgrounds on the Imd-triggered immune response, we determined the transcript levels of Relish and the Relish-dependent AMPs, dipterin A, cecropin C and attacin D, as well as drosomycin, which is induced primarily via the Toll pathway. We found that JNK activity is required for the early activation of Relish, since no induction was observed at 1 h post-infection in flies over-expressing dominant-negative Basket (Fig. 7 D). In contrast, JNK was found to have dampening effects on subsequent Relish up-regulation later, since Relish levels continued to rise after 4 h in the *dprx5* mutant background (Fig. 7 D). Consistent with previous results (Fig. 2 A), the induction of CecC and AttD, but not Dipt or Drs, was more pronounced in the *dprx5* mutant (Fig. 7 E–H). The effects were further enhanced by over-expression of Bsk^{DN}, although the dominant-negative Basket alone did not have any effects on the expression of these AMPs. We also found that JNK signaling is needed for more robust induction of Drs (Fig. 7 H).

3.6. dPrx5 is up-regulated in response to septic injury

The above data indicated that dPrx5 acts as a negative regulator of the immune response to infection. It is plausible then that activation of pathway(s) involved in regulating immunity could serve to induce dPrx5 expression, which through feedback inhibition could result in a more controlled immune response. Indeed we found that there was an increase in dPrx5 protein and mRNA levels (not shown) after septic injury with different bacteria, which became particularly evident after 5 h post-infection (Fig. 8).

4. Discussion

In this study we showed that *Drosophila* peroxiredoxin 5 is involved in negative control of the immune response, and furthermore its action has been tracked to the JNK signaling pathway.

The *dprx5* mutant was found to exhibit enhanced resistance to bacteria that primarily activate the Imd immune pathway. Several lines of evidence support this conclusion: *i*) downstream effectors of the immune response associated with the Imd pathway, particularly attacins and cecropins were clearly up-regulated in the absence of dPrx5 (Figs. 2 A and 7 F–G); *ii*) the *dprx5* mutant was cleared from the *E. coli* sepsis significantly faster (Fig. 1 E); and *iii*) the mortality of the mutant, infected with *B. subtilis* or *P. aeruginosa*, was reduced. The reduced mortality rate was particularly evident during the first 1–2 days after infection (Fig. 1 B and C). At the same time, production of AMPs in the mutant was enhanced and likely prevented bacterial proliferation during early stages of infection. To evade host defense, some bacteria, such as *P. aeruginosa*, employ suppression of AMP production early during infection [41]. The initial AMP deficit appeared to be compensated in the *dprx5* mutant. Furthermore, the elevated levels of attacins, cecropins and dipterocins, which display antimicrobial activity to a wide spectrum of both Gram-positive and Gram-negative bacteria [42–44], seem to underlie the enhanced resistance in the *dprx5* mutant.

While being relatively resistant to microbial challenge, the *dprx5* mutant is highly susceptible to oxidative stress caused by hydrogen peroxide or paraquat, as was reported previously [19]. Similarly, dPrx5 overexpression conferred greater resistance to oxidative stress but enhanced significantly susceptibility to bacterial infection (Fig. 1 D), suggesting a trade-off between dPrx5 immune and antioxidant functions.

A series of molecular and genetic approaches were used to investigate mechanisms by which dPrx5 controls the immune response. We determined that the activity of Relish was still required for the induction of the Imd-dependent AMPs (Fig. 3). The levels of AttD, an indicator for the Imd/Relish pathway, were significantly reduced in the *rel* mutant as well as mutants for the IKK complex and Dredd caspase, genes that are required for processing and activation of Relish [36,37]. When any of these mutants were placed in a *dprx5* background the AttD levels remained low. On the other hand, AttD suppression was partially compensated when *dtak1* mutation was combined with the *dprx5* mutation. dTak1 controls activation of both Imd and JNK-mediated immune response [11,13,14,21], which led us to suggest that dPrx5 could have negative control over activation of Relish-dependent genes by modulating the JNK branch of immune signaling. Indeed, we found that temporal patterns of activation of the JNK pathway, specifically phosphorylation of the JNK kinase Basket and transcriptional profiling of the downstream target genes, *puckered* and *ets21C*, were altered accordingly in the *dprx5* mutant (Figs. 4 and 5). We have also found that dPrx5 may control local peroxide levels, thus perhaps modulating the redox state of the dTak1-JNK kinase partners and coupled phosphatases, which in turn impacts kinase function. Enhanced phosphorylation of the JNK kinase Basket and the altered transcriptional profile of the JNK downstream targets (Fig. 6) in flies expressing redox-negative dPrx5 strongly suggest that dPrx5 regulates the dTak1-JNK arm of immune signaling by virtue of its peroxidase activity.

Altogether, the data placed dPrx5 downstream or at the level of dTak1 but upstream of Basket. This is supported by recent evidence that both JNK and the Imd pathways integrate to positively control the induction of AMPs [11]. This runs counter to a study conducted by Kim *et al.*, where RNAi and over-expression in S2 cells revealed a negative crosstalk between the JNK and Imd pathways, in which the JNK pathway was required for shutting down activation of AttA, triggered via the Imd pathway [12]. At present we attribute this discrepancy to the greater level of complexity of the immune response in the whole organism, which relies on the fat body in addition to a hemocyte-based subset of the immunity in contrast to the immune response in macrophage-like S2 cells.

There are two current models of Imd-mediated immune signaling in response to infection. An earlier model, initially suggested by Brennan and Anderson [45] places dTak1 within the Imd

pathway downstream of IMD and upstream of IKK complex and is based on the finding that the loss of dTak1 activity can block activation of the AMPs, elicited by IMD over-expression [21]. A second model, proposed by Delaney *et al.* and Kleino [11,46], places dTak1 upstream of JNK, rather than IKK. According to their data, activation of JNK signaling could rescue AMP expression from the loss of dTak1, while suppression of JNK signaling results in inhibition of Relish-dependent antimicrobial peptides. Our data favor the latter model, where the loss of dPrx5 activity results in activation of the JNK kinase Basket and transient response genes and positively controls the induction of Relish-dependent AMPs.

It is interesting that JNK was also required for the early activation of Relish at the transcriptional level, as Rel induction was significantly abrogated in Bsk^{DN} flies (Fig. 7 D). However, expression of Rel or over-expression of Bsk^{DN} did not seem to impact activation of the Rel-dependent AMPs, Dipt, Cec and Att, but did affect the Toll-dependent Drs (Fig. 7 H). It appears that constitutively produced amounts of Relish protein are sufficient for activation of the Imd/Relish-mediated response, where Relish processing and nuclear transport permit induction of the effector AMP genes. Nevertheless, differences in Relish mRNA levels in response to infection, that were also observed between control and *dprx5* mutant flies, suggest that Relish protein may play another function in modulating the immune response. Consistent with other reports [11], the patterns of Drs response also suggest that JNK is required for the Toll-mediated response and that dPrx5 acts upstream of the JNK kinase Basket (Fig. 7 H).

The proposed model does not fully explain the observed differences in the activation of Relish-dependent AMPs, nor the synergistic effects on AttD and CecC induction, observed in flies producing the dominant-negative Basket in the *dprx5* mutant background (Fig. 7 E–G). The analysis of Dipt, CecC and AttD promoters using AliBaba2 software (www.gene-regulation.com) did not reveal obvious differences that might affect the induction of attacin, cecropin and dipterin. Unlike Dipt, transcription of AttD and CecC could also require additional modifications of Relish, as suggested [47]. On the other hand, alterations in JNK signaling may in its turn cause changes in the transcription factors required for proper control of different AMPs. The differential responses of Dipt, CecC and AttD (Fig. 7) support this hypothesis. We also cannot exclude that, in addition to affecting phosphorylation of JNK (Basket), dPrx5 may also function downstream of JNK and directly affect activity of transcription factors or transcription complexes involved in regulating expression of different AMPs. Our previous finding that dPrx5 has multiple subcellular localizations, including nuclear, supports this notion. Possible interactions of a nuclear-localized form of dPrx5 with transcription complexes have been reported [39].

The dPrx5 itself is up-regulated in response to septic injury by different bacteria, both Gram-positive and Gram-negative. It is known that both injury and bacterial challenges cause release of ROS, which is either a component of cellular immune response to kill invaders or a downstream event of cell receptor-ligand interactions, which facilitates cell signaling. On the other hand, the excess of ROS, released in response to infection, may cause damage to the host cells. Thus, the up-regulation of dPrx5 after bacterial challenge could only be a response to the elevated ROS levels and serve a protective role to host cells during the immune response, at a time when excess reactive oxygen species are released. However, we did not find evidence that dPrx5 is up-regulated in response to oxidants, including paraquat or hydrogen peroxide; nor did we find any evidence for the presence of ARE (antioxidant response element) in the dPrx5 promoter [19]. Besides, adult flies without dPrx5 seem to survive better during infection than controls and transgenic flies that express dPrx5 at higher levels (Fig. 1). Thus, any protective role that dPrx5 plays in the immune response would appear to be minor. Given the role of dPrx5 in suppressing the immune response, its up-regulation by bacterial infection may instead represent a negative feedback mechanism, quenching an excessive immune response. The presence of a κB binding motif in the *dprx5* gene promoter [19] supports this hypothesis.

In summary, we have determined that one of the biological functions of peroxiredoxin 5 is to control the immune response. To date, the role of antioxidants, such as peroxiredoxins, during infection has been generally described as protective in nature, where peroxiredoxins scavenge excessive ROS that are released by cells in order to kill microbial invaders. Our findings, however, suggest that peroxiredoxins may function to dampen the activation of the immune response, acting as negative regulators of specific immune pathways. The results of the present research placed dPrx5 at the dTak1-JNK branch of immune signaling, although the data suggest that dPrx5 may also act downstream or in parallel to JNK. The latter conjecture is currently under investigation.

Acknowledgments

We thank Drs. J. Abrams, D. Bohmann, B. Rogina and N. Silverman for providing fly strains, Dr. C. Buchanan for providing bacterial strains and Drs. R. Jones and J. Waddle (SMU) for a critical reading of the manuscript. This work was supported by grant R21 AG025096-01 from the National Institute on Aging/National Institutes of Health.

Abbreviations

Prx	peroxiredoxin
AMP	antimicrobial peptide
Att	attacin
Cec	cecropin
Dipt	diphtericin, Drs, drosomycin
Mtk	metchnikov
Imd	immune deficiency
JNK	c-Jun N-terminal kinase

References

- [1]. Shau H, Kim AT, Hedrick CC, Lulis AJ, Tompkins C, Finney R, Leung DW, Paglia DE. Endogenous natural killer enhancing factor-B increases cellular resistance to oxidative stresses. *Free Radic Biol Med* 1997;22:497–507. [PubMed: 8981042]
- [2]. Jin DY, Chae HZ, Rhee SG, Jeang KT. Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation. *J Biol Chem* 1997;272:30952–30961. [PubMed: 9388242]
- [3]. Hansen JM, Moriarty-Craige S, Jones DP. Nuclear and cytoplasmic peroxiredoxin-1 differentially regulate NF-kappaB activities. *Free Radic Biol Med* 2007;43:282–288. [PubMed: 17603937]
- [4]. Kim YJ, Lee WS, Ip C, Chae HZ, Park EM, Park YM. Prx1 suppresses radiation-induced c-Jun NH2-terminal kinase signaling in lung cancer cells through interaction with the glutathione S-transferase Pi/c-Jun NH2-terminal kinase complex. *Cancer Res* 2006;66:7136–7142. [PubMed: 16849559]
- [5]. Haridas V, Ni J, Meager A, Su J, Yu GL, Zhai Y, Kyaw H, Akama KT, Hu J, Van Eldik LJ, Aggarwal BB. TRANK, a novel cytokine that activates NF-kappa B and c-Jun N-terminal kinase. *J Immunol* 1998;161:1–6. [PubMed: 9647199]
- [6]. Jung H, Kim T, Chae HZ, Kim KT, Ha H. Regulation of macrophage migration inhibitory factor and thiol-specific antioxidant protein PAG by direct interaction. *J Biol Chem* 2001;276:15504–15510. [PubMed: 11297517]
- [7]. Kang SW, Chang TS, Lee TH, Kim ES, Yu DY, Rhee SG. Cytosolic peroxiredoxin attenuates the activation of Jnk and p38 but potentiates that of Erk in HeLa cells stimulated with tumor necrosis factor-alpha. *J Biol Chem* 2004;279:2535–2543. [PubMed: 14597634]
- [8]. Veal EA, Findlay VJ, Day AM, Bozonet SM, Evans JM, Quinn J, Morgan BA. A 2-Cys peroxiredoxin regulates peroxide-induced oxidation and activation of a stress-activated MAP kinase. *Mol Cell* 2004;15:129–139. [PubMed: 15225554]

- [9]. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* 1996;86:973–983. [PubMed: 8808632]
- [10]. Lemaitre B, Reichhart JM, Hoffmann JA. *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc Natl Acad Sci U S A* 1997;94:14614–14619. [PubMed: 9405661]
- [11]. Delaney JR, Stoven S, Uvell H, Anderson KV, Engstrom Y, Mlodzik M. Cooperative control of *Drosophila* immune responses by the JNK and NF-kappaB signaling pathways. *Embo J* 2006;25:3068–3077. [PubMed: 16763552]
- [12]. Kim T, Yoon J, Cho H, Lee WB, Kim J, Song YH, Kim SN, Yoon JH, Kim-Ha J, Kim YJ. Downregulation of lipopolysaccharide response in *Drosophila* by negative crosstalk between the AP1 and NF-kappaB signaling modules. *Nat Immunol* 2005;6:211–218. [PubMed: 15640802]
- [13]. Silverman N, Zhou R, Erlich RL, Hunter M, Bernstein E, Schneider D, Maniatis T. Immune activation of NF-kappaB and JNK requires *Drosophila* TAK1. *J Biol Chem* 2003;278:48928–48934. [PubMed: 14519762]
- [14]. Kallio J, Leinonen A, Ulvila J, Valanne S, Ezekowitz RA, Ramet M. Functional analysis of immune response genes in *Drosophila* identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells. *Microbes Infect* 2005;7:811–819. [PubMed: 15890554]
- [15]. Boutros M, Agaisse H, Perrimon N. Sequential activation of signaling pathways during innate immune responses in *Drosophila*. *Dev Cell* 2002;3:711–722. [PubMed: 12431377]
- [16]. Kim M, Lee JH, Lee SY, Kim E, Chung J. Caspar, a suppressor of antibacterial immunity in *Drosophila*. *Proc Natl Acad Sci U S A* 2006;103:16358–16363. [PubMed: 17050695]
- [17]. Tsuda M, Langmann C, Harden N, Aigaki T. The RING-finger scaffold protein Plenty of SH3s targets TAK1 to control immunity signalling in *Drosophila*. *EMBO Rep* 2005;6:1082–1087. [PubMed: 16179944]
- [18]. Kim LK, Choi UY, Cho HS, Lee JS, Lee WB, Kim J, Jeong K, Shim J, Kim-Ha J, Kim YJ. Down-regulation of NF-kappaB target genes by the AP-1 and STAT complex during the innate immune response in *Drosophila*. *PLoS Biol* 2007;5:e238. [PubMed: 17803358]
- [19]. Radyuk SN, Michalak K, Klichko VI, Benes J, Rebrin I, Sohal RS, Orr WC. Peroxiredoxin 5 confers protection against oxidative stress and apoptosis and also promotes longevity in *Drosophila*. *Biochem J* 2009;419:437–445. [PubMed: 19128239]
- [20]. Rutschmann S, Jung AC, Zhou R, Silverman N, Hoffmann JA, Ferrandon D. Role of *Drosophila* IKK gamma in a toll-independent antibacterial immune response. *Nat Immunol* 2000;1:342–347. [PubMed: 11017107]
- [21]. Vidal S, Khush RS, Leulier F, Tzou P, Nakamura M, Lemaitre B. Mutations in the *Drosophila* dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses. *Genes Dev* 2001;15:1900–1912. [PubMed: 11485985]
- [22]. Leulier F, Rodriguez A, Khush RS, Abrams JM, Lemaitre B. The *Drosophila* caspase Dredd is required to resist gram-negative bacterial infection. *EMBO Rep* 2000;1:353–358. [PubMed: 11269502]
- [23]. Hedengren M, Asling B, Dushay MS, Ando I, Ekengren S, Wihlborg M, Hultmark D. Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. *Mol Cell* 1999;4:827–837. [PubMed: 10619029]
- [24]. Michalak K, Orr WC, Radyuk SN. *Drosophila* peroxiredoxin 5 is the second gene in a dicistronic operon. *Biochem Biophys Res Commun* 2008;368:273–278. [PubMed: 18211821]
- [25]. Radyuk SN, Sohal RS, Orr WC. Thioredoxin peroxidases can foster cytoprotection or cell death in response to different stressors: over- and under-expression of thioredoxin peroxidase in *Drosophila* cells. *Biochem J* 2003;371:743–752. [PubMed: 12556226]
- [26]. Flatt T, Heyland A, Rus F, Porpiglia E, Sherlock C, Yamamoto R, Garbuzov A, Palli SR, Tatar M, Silverman N. Hormonal regulation of the humoral innate immune response in *Drosophila melanogaster*. *J Exp Biol* 2008;211:2712–2724. [PubMed: 18689425]
- [27]. Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. *Annu Rev Immunol* 2007;25:697–743. [PubMed: 17201680]

- [28]. Hedengren-Olcott M, Olcott MC, Mooney DT, Ekengren S, Geller BL, Taylor BJ. Differential activation of the NF-kappaB-like factors Relish and Dif in *Drosophila melanogaster* by fungi and Gram-positive bacteria. *J Biol Chem* 2004;279:21121–21127. [PubMed: 14985331]
- [29]. Lau GW, Goumnerov BC, Walendziewicz CL, Hewitson J, Xiao W, Mahajan-Miklos S, Tompkins RG, Perkins LA, Rahme LG. The *Drosophila melanogaster* toll pathway participates in resistance to infection by the gram-negative human pathogen *Pseudomonas aeruginosa*. *Infect Immun* 2003;71:4059–4066. [PubMed: 12819096]
- [30]. De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *EMBO J* 2002;21:2568–2579. [PubMed: 12032070]
- [31]. Choe KM, Lee H, Anderson KV. *Drosophila* peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor. *Proc Natl Acad Sci U S A* 2005;102:1122–1126. [PubMed: 15657141]
- [32]. Takehana A, Yano T, Mita S, Kotani A, Oshima Y, Kurata S. Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in *Drosophila* immunity. *Embo J* 2004;23:4690–4700. [PubMed: 15538387]
- [33]. Georgel P, Naitza S, Kappler C, Ferrandon D, Zachary D, Swimmer C, Kopczynski C, Duyk G, Reichhart JM, Hoffmann JA. *Drosophila* immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis. *Dev Cell* 2001;1:503–514. [PubMed: 11703941]
- [34]. Naitza S, Rosse C, Kappler C, Georgel P, Belvin M, Gubb D, Camonis J, Hoffmann JA, Reichhart JM. The *Drosophila* immune defense against gram-negative infection requires the death protein dFADD. *Immunity* 2002;17:575–581. [PubMed: 12433364]
- [35]. Silverman N, Zhou R, Stoven S, Pandey N, Hultmark D, Maniatis T. A *Drosophila* IkappaB kinase complex required for Relish cleavage and antibacterial immunity. *Genes Dev* 2000;14:2461–2471. [PubMed: 11018014]
- [36]. Stoven S, Silverman N, Junell A, Hedengren-Olcott M, Erturk D, Engstrom Y, Maniatis T, Hultmark D. Caspase-mediated processing of the *Drosophila* NF-kappaB factor Relish. *Proc Natl Acad Sci U S A* 2003;100:5991–5996. [PubMed: 12732719]
- [37]. Stoven S, Ando I, Kadalayil L, Engstrom Y, Hultmark D. Activation of the *Drosophila* NF-kappaB factor Relish by rapid endoproteolytic cleavage. *EMBO Rep* 2000;1:347–352. [PubMed: 11269501]
- [38]. Johansson KC, Metzendorf C, Soderhall K. Microarray analysis of immune challenged *Drosophila* hemocytes. *Exp Cell Res* 2005;305:145–155. [PubMed: 15777795]
- [39]. Kropotov AV, Grudinkin PS, Pleskach NM, Gavrilov BA, Tomilin NV, Zhivotovsky B. Downregulation of peroxiredoxin V stimulates formation of etoposide-induced double-strand DNA breaks. *FEBS Lett* 2004;572:75–79. [PubMed: 15304327]
- [40]. Adachi-Yamada T, Nakamura M, Irie K, Tomoyasu Y, Sano Y, Mori E, Goto S, Ueno N, Nishida Y, Matsumoto K. p38 mitogen-activated protein kinase can be involved in transforming growth factor beta superfamily signal transduction in *Drosophila* wing morphogenesis. *Mol Cell Biol* 1999;19:2322–2329. [PubMed: 10022918]
- [41]. Apidianakis Y, Mindrinos MN, Xiao W, Lau GW, Baldini RL, Davis RW, Rahme LG. Profiling early infection responses: *Pseudomonas aeruginosa* eludes host defenses by suppressing antimicrobial peptide gene expression. *Proc Natl Acad Sci U S A* 2005;102:2573–2578. [PubMed: 15695583]
- [42]. Samakovlis C, Kimbrell DA, Kylsten P, Engstrom A, Hultmark D. The immune response in *Drosophila*: pattern of cecropin expression and biological activity. *EMBO J* 1990;9:2969–2976. [PubMed: 2390977]
- [43]. Tryselius Y, Samakovlis C, Kimbrell DA, Hultmark D. CecC, a cecropin gene expressed during metamorphosis in *Drosophila* pupae. *Eur J Biochem* 1992;204:395–399. [PubMed: 1740152]
- [44]. Winans KA, King DS, Rao VR, Bertozzi CR. A chemically synthesized version of the insect antibacterial glycopeptide, dipteracin, disrupts bacterial membrane integrity. *Biochemistry* 1999;38:11700–11710. [PubMed: 10512626]
- [45]. Brennan CA, Anderson KV. *Drosophila*: the genetics of innate immune recognition and response. *Annu Rev Immunol* 2004;22:457–483. [PubMed: 15032585]

- [46]. Kleino A, Valanne S, Ulvila J, Kallio J, Myllymaki H, Enwald H, Stoven S, Poidevin M, Ueda R, Hultmark D, Lemaitre B, Ramet M. Inhibitor of apoptosis 2 and TAK1-binding protein are components of the Drosophila Imd pathway. *Embo J* 2005;24:3423–3434. [PubMed: 16163390]
- [47]. Wiklund ML, Steinert S, Junell A, Hultmark D, Stoven S. The N-terminal half of the Drosophila Rel/NF-kappaB factor Relish, REL-68, constitutively activates transcription of specific Relish target genes. *Dev Comp Immunol* 2009;33:690–696. [PubMed: 19135474]

Fig. 1. Survivorship of *dprx5* mutant after infection and clearance from bacteria

A minimum one hundred flies was used for each cohort. All flies were aged until day 10 followed by infection. The results of survivorship are pooled data of three-four independent experiments. **A**, **B**, **C** and **D**, a percentage of live flies is shown on the Y axis. **A**, *dprx5* mutant and control (*yw*) flies were exposed to a sporulating culture of *B. bassiana* followed by incubation at 25°C. **B**, *dprx5* mutant and control (*yw*) flies were septicely injured with *B. subtilis* and incubated at 25°C. Paired t test showed statistically significant differences in mean survivorship for *yw* versus *dprx5* infected with *B. subtilis* ($P=0.049$). **C** and **D**, *dprx5* mutant and control (*yw*) flies (**C**) or flies expressing dPrx5 from the UAS-dPrx5 transgene (**D**) were naturally infected with *P. aeruginosa* and incubated at 25°C. UAS-dPrx5 transgene was

expressed in flies with *dprx5* null background using ubiquitous Act-GAL4 driver (UAS-dPrx5/Act). The resulting genotype was *yw*; UAS-dPrx5/Act; *dprx5/dprx5* and the genotypes of corresponding controls were *yw*; UAS-dPrx5/+; *dprx5/dprx5* (UAS-dPrx5/+) and *yw*; +/Act; *dprx5/dprx5* (+/Act). Paired t test showed statistically significant differences in survivorship between infected flies expressing dPrx5 from transgene (UAS-dPrx5) and corresponding driver and transgene controls ($P=0.030$ and $P=0.027$) and marginally significant differences in mean survivorship between *dprx5* mutant and *y w* control ($P=0.055$). **E**, flies were septically injured with ampicillin-resistant strain of *E. coli* followed by bacteriological analysis of fly lysates at various time intervals after infection. At least ten flies were taken for each sample. The number of colonies per fly is indicated on the Y axis. The results are mean $\pm$ SD from two independent experiments and the statistically significant differences ($P<0.05$) are denoted by asterisks.

Fig. 2. AMP levels in flies infected with *B. bassiana* and *E. coli*

A, Control and mutant 10 days old flies were exposed to sporulating fungi and maintained for 24 h at 25°C or they were injured with a needle dipped in a *E. coli* culture followed held for 8 h at 25°C. RNA was extracted and AMP levels were then determined by real-time RT-PCR. The signals obtained for AMP genes were normalized with respect to signals obtained for house-keeping gene (rp49). Plotted are fold changes in the AMP levels in *dprx5* mutant compared to *yw* control. At least four independent determinations were performed for each AMP. More than one fold significant differences between the *dprx5* mutant relative to *yw* control were determined for AttD ($P=0.0455$) and CecC ($P=0.0148$), as denoted by asterisks.

B, 10 da old flies over-expressing dPrx5 (UAS-dPrx5/Act) and corresponding driver and

transgene controls (UAS-dPrx5/+ and +/-Act) were infected with *E. coli* by pricking. RNA was extracted and AMP levels were then determined by real-time RT-PCR. The signals obtained for AMP genes were normalized with respect to signals obtained for house-keeping gene (actin). The results are mean $\pm$ SD from three independent experiments and the statistically significant differences ($P < 0.05$) are denoted by asterisks. **C**, RNA was isolated from flies that were pricked with a needle dipped in *E. coli* suspension (*E.coli*) or the same suspension inactivated for 5 min by boiling (t°), as well as from flies pricked with a sterile needle (Injury). Relative to a housekeeping gene mRNA ratios are plotted on the Y axis.

Fig. 3. The immune response induced by bacterial infection is altered in mutants

Ten day old *y w* control (C), *dprx5*, *tak1*, *rel*^{E20}, *kenny* (*key1*), *ird5* and *dredd* mutant flies and double mutants underexpressing dTak1, Relish, genes comprising IKK complex (Kenny and Ird5), Dredd caspase and dPrx5 were infected with *E. coli* and incubated at 25°C for 6 hours. RNA was extracted and the transcriptional levels of attacin D as a read-out gene for the Imd/Relish pathway were determined. The results are mean $\pm$ SD from three independent experiments. Statistically significant differences ($P < 0.05$) were between *y w* control and the *dprx5* mutant, as well as mutants of the genes comprising the IMD/Relish axis, and double mutants. There were also significant differences between *dtak1* mutant and *dtak1;dprx5* double mutant.

Fig. 4. dPrx5 affects JNK signaling by signal-dependent phosphorylation of Basket

Proteins were isolated from control (*yw*), *dprx5* mutant and flies over-expressing dPrx5 (*UAS-dPrx5*) at different time intervals after infection with *E.coli* and resolved by PAGE electrophoresis. Immunoblot analysis was performed with anti-phos-JNK antibodies to detect phosphorylated JNK (Basket) and with anti-JNK antibodies as a control. Total protein levels were equal in each sample, as was determined after re-probing blots with anti-Actin antibodies (not shown). Protein molecular weight markers (kDa) are indicated on the left. Intensity of the phos-JNK signals standardized against signals obtained for JNK is plotted on the graphs. The results are mean $\pm$ SD, $n=3$.

Fig. 5. Expression analysis of transient response genes *pucker* (A), *ets21C* (B) and *relish* (C), induced by bacterial infection

Total RNA was isolated from *dprx5* mutant, control (*yw*) flies and flies over-expressing dPrx5 (UAS-dPrx5/Act) infected with *E. coli* at different time intervals. RT-PCR was performed with primers specific for Pucker, Ets21C and Relish, and signals obtained were standardized against signal obtained for the housekeeping gene rp49. Statistically significant ($P < 0.05$) were differences in the levels of Puc, Ets21C and Rel at 0 h and 30 min post-infection and also Rel at 1 h post-infection, as denoted by asterisks. Significant were also differences between control and flies over-expressing dPrx5 at 30 min post-infection (Puc), 1 h (Puc, Ets21C and Rel) and 2 h (Rel). The results are mean $\pm$ SD from three independent experiments.

Fig. 6. Effects of the wild type and redox-negative forms of dPrx5 on JNK signaling

The expression of dPrx5 or dPrx5^{RN} was achieved using corresponding UAS transgenes and *dprx5* or recombinant Da-GAL4, *dprx5* alleles. The genotype of the generated flies was *yw*; UAS-dPrx5 (or dPrx5^{RN})/+; *dprx5*/Da-GAL4, *dprx5*. **A** and **B**, expression analysis of *puckered* and *Ets21C*, induced by bacterial infection. Total RNA was isolated from flies infected with *E. coli* at different time intervals. RT-PCR was performed with primers specific for Puckered and Ets21C and signals obtained were standardized against signals obtained for the housekeeping gene *rp49*. Statistically significant ($P < 0.05$) differences are denoted by asterisks. **C**, Expression levels of wild type and redox-negative forms of dPrx5 protein are shown on the upper image. Phosphorylation of Basket in flies expressing wild type and redox-negative forms of dPrx5 are shown on the lower image. Proteins were isolated from control (*yw*), *dprx5* mutant (*dprx5*) and flies expressing UAS-dPrx5 or UAS-dPrx5^{RN} in *dprx5* null background (indicated as dPrx5 and dPrx5^{RN}) and resolved by PAGE electrophoresis. Immunoblot analysis was performed with anti-dPrx5 and anti-Actin antibodies to control for loading. Phosphorylated JNK (Basket) was detected with anti-phos-JNK antibodies and with anti-JNK antibodies as a control. Protein molecular weight markers (kDa) are indicated on the left. Intensity of the phos-JNK signals standardized against signals obtained for JNK is plotted on the graphs. The results are mean $\pm$ SD, $n=3$. Asterisks indicate statistically significant differences between the *dprx5* mutant or flies expressing dPrx5^{RN} and *yw* control and dPrx5-expressing flies.

Fig.7. Effects of dPrx5 and JNK signaling on the immune response genes

The genotype of the control (C) was UAS-Bsk^{DN}; S106/+;+/+ and the genotype of flies under-expressing dPrx5 (*dprx5*) was UAS-Bsk^{DN}; S106/+;*dprx5/dprx5*. Expression of dominant-negative Basket was achieved by feeding one week-old flies food containing 0.1 mg/ml mifepristone dissolved in ethanol (Bsk^{DN}) or ethanol-only added food as a control (–). Food with mifepristone was changed daily. **A**, Analysis of Basket expression. After two days of feeding mifepristone, RNA was isolated followed by real time RT-PCR analysis with primers specific for Basket. **B–H**, After feeding for two days food with or without mifepristone, flies were infected with 24 h broth culture of *P. aeruginosa* by pricking and incubated at 25°C for 0 h, 1 h and 4 h followed by RNA extraction. RT-PCR was performed with primers specific for Puckered (**B**), Ets21C (**C**), Relish (**D**), Dipt (**E**), CecC (**F**), AttD (**G**) and Drs (**H**) and the gene-specific signals were standardized against signals obtained for the housekeeping gene *rp49*. Statistically significant ($P < 0.05$) were differences in the levels of Puc, Ets21C and Rel between control flies (no mifepristone) and flies expressing Bsk^{DN} in normal (C) or *dprx5* background at 1 h after infection, as denoted by asterisks. Significant differences were also in the levels of CecC between *dprx5* mutant and control, expressing dominant-negative Basket at 1 h and 4 h post-infection; AttD at 4 h post-infection between control and the *dprx5* mutant expressing or not expressing Bsk^{DN}, as well as Drs between flies with wild type or dominant-negative Basket, as denoted by asterisks. All RT-PCR results are mean $\pm$ SD from three-four determinations for each of two independent experiments.

Fig. 8. dPrx5 response to infection

Control and *dprx5* mutant flies were septically injured with *B. subtilis*, *M. luteus*, *P. aeruginosa*, *S. marcescens* or *E. coli* suspensions or with a sterile needle and kept for 2 h and 5 h at 25°C. At least ten flies were taken for each sample. Proteins were extracted, loaded in the amounts of 5 µg and resolved by PAGE, followed by immunoblot analysis using anti-dPrx5 antibodies and anti-actin antibodies to control for a loading. Protein molecular weight markers (kDa) are indicated on the left. Ratios of dPrx5 to actin signals are plotted on the Y axis. The results are mean ± SD from three determinations. Significant were differences between non-infected flies and flies infected with bacteria, as indicated by asterisks.

Table I

Primers used for the real time RT-PCR analysis of gene expression.

Gene	Primer name and structure.	
Basket	Forward	5' CCGCTTACGATACTATCACC
	Reverse	5' GACCAGGTAGACATCCTGA
Immune response deficient 5	Forward	5' GGCTAGACATCTGCCCAAGTC
	Reverse	5' CCATCGCTGCGCTTAGCTC
Puckered	Forward	5' GCAGAATTTGCGCAAGAGCGG
	Reverse	5' GAGCAGTTACTACCCGCCAG
Relish	Forward	5' ATCCGGAGAAGGGAGAACTGG
	Reverse	5' CAGTAGGGCGTAAGCCTGTG
Ets21C	Forward	5' ATCCAGCTGTGGCAGTTTCT
	Reverse	5' TGCACCTTGGTCATGATGTT
Attacin A+B	Forward	5' ACCAGAGATTAGCACGTCCATCC
	Reverse	5' <i>FAM</i> -CGTTGACTGGATTACTCCCACATC
Attacin C	Forward	5' GTCAGTTCCAGGCCGTGTCC
	Reverse	5' <i>FAM</i> -CGTCCACTCCCGTAACCAG
Attacin D	Forward	5' CGTTGAGGTTGAGATTGCCACT
	Reverse	5' <i>FAM</i> -CGGTCCCTCAGTTCGGCATGAC
Cecropin C	Forward	5' CCAATGCGCTCGATTCTCTTG
	Reverse	5' <i>FAM</i> -CGATTCCATCAGCATTGGACAAT
Diptericin	Forward	5' CCACCGCAGTACCACTCAAT
	Reverse	5' <i>FAM</i> -CGATGACTGCAAAGCCAAAACCA
Drosocin	Forward	5' AGTGAGGCGCGAGGCACTG
	Reverse	5' CATCAGATGGGTGGTGGCCTGA
Defensin	Forward	5' CGCATAGAAGCGAGCCACATG
	Reverse	5' GCAGTAGCCGCCTTTGAACC
Drosomycin	Forward	5' GCTGTCCTGATGCTGGTGGT
	Reverse	5' <i>FAM</i> -CGGAAAGGGACCCTTGATCTTC
Metchnikowin	Forward	5' CAGTGCTGGCAGAGCCTCAT
	Reverse	5' <i>FAM</i> -CGTCGGTTAGGATTGAAGGGCGA