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Short Article

Infection-Induced Intestinal Oxidative Stress Triggers Organ-to-Organ Immunological Communication in *Drosophila*

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SUMMARY

Local infections can trigger immune responses in distant organs, and this interorgan immunological crosstalk helps maintain immune homeostasis. We find that enterobacterial infection or chemically and genetically stimulating reactive oxygen species (ROS)-induced stress responses in the *Drosophila* gut triggers global antimicrobial peptide (AMP) responses in the fat body, a major immune organ in flies. ROS stress induces nitric oxide (NO) production in the gut, which triggers production of the AMP *Diptericin*, but not *Drosomycin*, in the fat body. Hemocytes serve as a signaling relay for communication between intestinal ROS/NO signaling and fat body AMP responses. The induction of AMP responses requires Rel/NF-κB activation within the fat body. Although Rel-mediated *Drosomycin* induction is repressed by the AP-1 transcription factor, this repressor activity is inhibited by intestinal ROS. Thus, intestinal ROS signaling plays an important role in initiating gut-to-fat body immunological communication in *Drosophila*.

INTRODUCTION

Innate immunity, which is evolutionarily conserved in plants and animals, is the first line of the host defense. Several key signaling molecules in this field, which helped discover Toll-like receptors in mammals, were identified using the *Drosophila* model system (Lemaitre et al., 1996; Medzhitov et al., 1997; Poltorak et al., 1998; Rosetto et al., 1995). Genetic studies in *Drosophila* have revealed that septic injury with Gram-positive bacteria or fungi can predominately stimulate Dif/Dorsal in Toll pathway to express *Drosomycin* (*Drs*) antimicrobial peptide (AMP), while the infection with Gram-negative bacteria stimulates Rel in IMD pathway to express *Diptericin* (*Dpt*) AMP. However, the innate immune response to oral infection is substantially different to septic injury. For example, bacteria oral infection of larvae resulted in Rel transcription activity to become manifested in expressing global AMPs (Basset et al., 2000; Vidal et al., 2001). Some studies suggested that Rel may act alone or become a heterodimer with Dif or Dorsal in inducing AMP responses (Han and Ip, 1999; Hedengren et al., 1999).

Reactive oxygen species (ROS) are also highly efficient immune effector molecules, which exert broad-spectrum microbicidal activity in clearing microbes from the gut (Ha et al., 2005). Intestinal ROS are generated by dual oxidase (DUOX) upon gut-microbe contact, by which the enzyme activity and transcription level of DUOX are elevated (Ha et al., 2009). However, ROS are destructive to gut mucosa cells (Buchon et al., 2009) and therefore the action must be transient. For full host protection, the gut immune response must subsequently trigger other systemic immune responses in distinct tissues/organs to clear the remaining pathogens escaping from the intestine.

Although individual organs in the body are designated to perform their respective functions, they do not work alone without interaction with the others. Interorgan communication helps one organ adjust its performance based on another organ's response to physiological or pathological conditions. Thus, local disturbance of an organ's functional homeostasis can lead to a systemic response affecting remote organs. For instance, inflammatory bowel diseases caused by commensal microorganisms may result in a number of extra-intestinal inflammatory disorders, including arthritis and inflammation of the liver and eye (Macdonald and Monteleone, 2005).

The molecular mechanisms underlying the organ-to-organ innate immune communication remain unclear, however. Interestingly, intestinal nitric oxide (NO) has been previously shown to function as an inducer for the interorgan immune communication in *Drosophila*. Induction of NO production in the gut of uninfected larvae triggers systemic immune responses (Foley and O'Farrell, 2003) similar to those induced by *Ecc15* (*Erwinia carotovora subsp.*) oral infection (Basset et al., 2000). Another reported signaling inducer involved in interorgan communication is a monomeric PGN fragment known as terminal monomer tracheal cytotoxin, which can cross the gut barrier into the hemolymph and subsequently trigger a systemic immune response in the fat body (Zaidman-Rémy et al., 2006).

Although intestinal ROS have long been recognized for their roles in the onset of inflammatory bowel disease (Rezaie et al., 2007), it remains largely unknown whether intestinal ROS also triggers interorgan immunological communication. We used

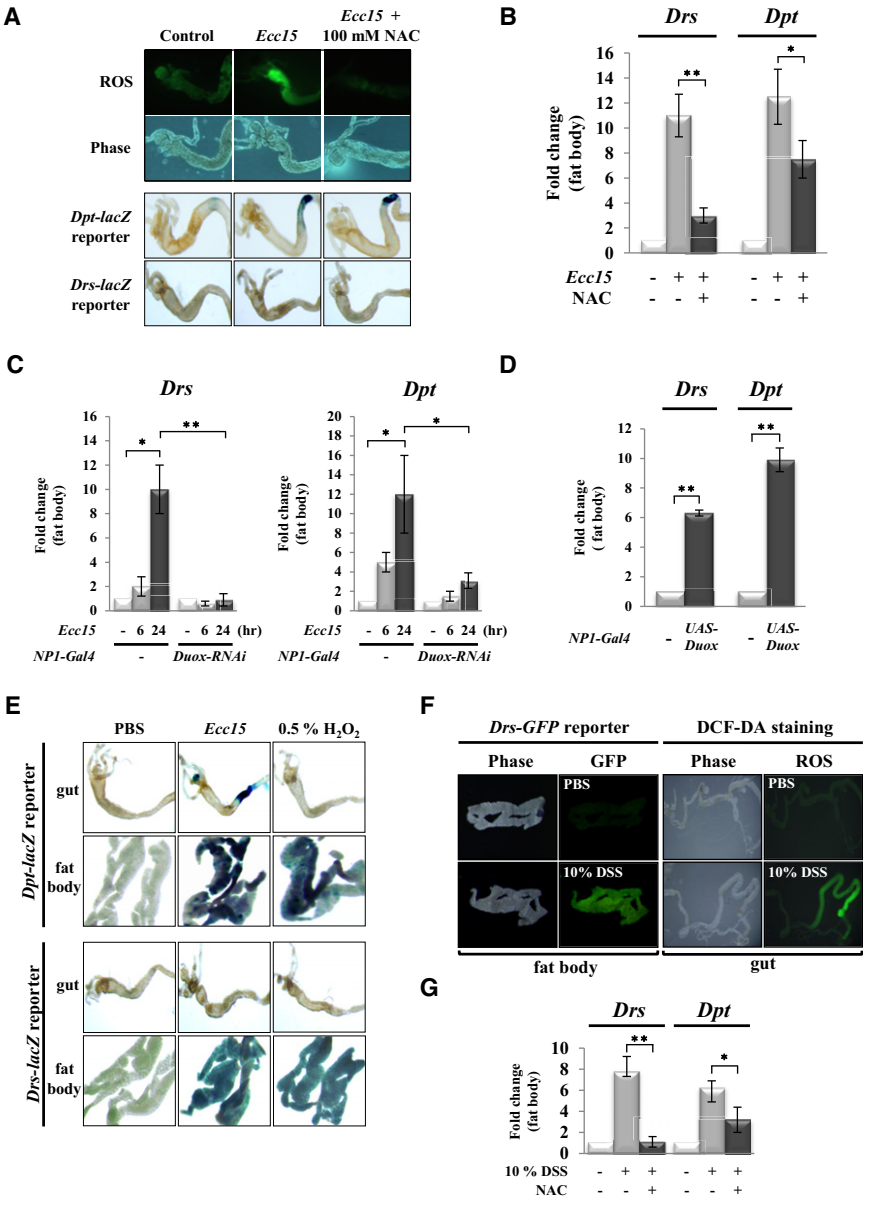

Figure 1. Stimulation of Intestinal Oxidative Stress, by Infection, Genetic Means, or Pro-oxidants, Triggers a Gut-to-Fat Body Immunological Communication

(A) ROS-induced DCF-DA fluorescence signal and AMP reporter expression in the gut. Larvae orally infected with *Ecc15* for 6 hr at RT in the presence or absence of 100 mM NAC were subsequently fed with DCF-DA for 3 hr. The fluorescent signal (green) in the *Ecc15*-infected gut was diminished after ingestion of NAC. β-gal staining (blue) of *Dpt-lacZ* or *Drs-lacZ* reporter in the *Ecc15*-fed larval gut. The *Ecc15*-induced *Dpt-lacZ* signal was not notably affected by the NAC.

(B) NAC ingestion suppresses the AMP responses in the fat body of *Ecc15*-fed larvae. Shown is qRT-PCR analysis of *Drs* and *Dpt* expressions in the fat body of larvae cofed *Ecc15* with or without 100 mM NAC for 24 hr.

(C) Knocking down intestinal *Duox* decreases AMP responses in the fat body of larvae fed *Ecc15*. Shown is qRT-PCR analysis of *Drs* and *Dpt* expressions in the fat body of *NP1-Gal4 > UAS-Duox-RNAi* versus *NP1-Gal4* larvae that ingested *Ecc15* or PBS as control.

(D) Overexpressing *Duox* in gut triggers global AMP responses in the fat body of larvae without infection. Shown is qRT-PCR analysis of *Dpt* and *Drs* expressions in fat body of transgenic larvae carrying *NP1-Gal4 > UAS-Duox*.

(E) H_2O_2 ingestion also induces AMP responses in the fat body. Shown is β-gal staining of gut and fat body in the *Drs-lacZ* or *Dpt-lacZ* transgenic larvae that ingested 0.5% H_2O_2, *Ecc15*, or PBS diets for 12 or 24 hr.

(F) Fluorescent microscopic image of *Drs-GFP* reporter expression in the fat body and DCF-DA fluorescence in the gut of larvae that ingested 10% DSS for 36 hr (*Drs-GFP* reporter) or 24 hr (DCF-DA staining).

(G) DSS-induced endogenous *Drs* and *Dpt* expressions in the fat body are also diminished by larvae cofed with 100 mM NAC. Results in (B)–(D) and (G) are normalized to the levels of *rp49* and represented as mean ± SD from three independent experiments.

Drosophila, an excellent genetic model system, to perform in vivo and genetic experiments in a study of the possible role of intestinal ROS in the triggering of gut-to-fat body interorgan innate immune communication.

RESULTS

Stimulation of Intestinal ROS Triggers a Gut-to-Fat Body Immunological Communication

Enterobacteria *Ecc15* oral infection has been shown to locally induce a specific AMP response (e.g., *Dpt* expression) and ROS stress in adult gut (Lemaitre and Hoffmann, 2007). Intriguingly, although *Ecc15* is mostly undetectable in the hemolymph of larvae orally infected with *Ecc15* (Basset et al., 2000), the infection also triggers global AMP responses in remote organs such as the fat body and the tracheal system (Basset et al., 2000; Tzou et al., 2000). However, it is unclear how such an organ-to-organ immunological communication is regulated. Thus, we asked whether one local organ and one distinct organ's AMP response to *Ecc15* oral infection would be ROS dependent. As larvae are almost constantly consuming food, this makes the larval stage an ideal time to study oral infection-induced immunological communication. When we fed the larvae *Ecc15*, we found, as expected, an increase in production of ROS and an increase in *Dpt-lacZ* transgene expression in the gut. However, inhibition of ROS production by a ROS scavenger-NAC did not notably affect the *Dpt-lacZ* expression (Figure 1A), as has been previously reported in one study of adult gut (Ha et al., 2005). These results prompted us to investigate whether stimulation of intestinal ROS triggers a specific organ-to-organ immunological communication. Because the fat body, analogous to a liver in *Drosophila*, is a major immune system organ in larvae of this

insect, we focused on its AMP response to intestinal ROS. Surprisingly, in contrast to that observed in gut, our study found that NAC markedly suppressed the microbe-induced global AMP responses in fat body, as evidenced by the expressions of endogenous *Drs* and *Dpt* (Figure 1B), suggesting that the *Ecc15*-induced oxidative stress was critical in regulating the AMP responses of the fat body, but not locally in the gut. To avoid the nonspecific effects of NAC, we genetically attenuated the intestinal ROS production by silencing *Duox*, a *Drosophila* NADPH oxidase homolog, by crossing a *Duox-RNAi* transgenic line with a gut-specific driver (*NP1-Gal4*). As was found with NAC, the *Ecc15*-induced global AMP responses in the fat body were suppressed (Figure 1C).

Innate immunity can also be triggered without microbial infection (Rock et al., 2010), as is the case when localized DNA damage in the *Drosophila* epidermis induces an innate immune response (Karpac et al., 2011). Therefore, we used genetic and chemical means to induce intestinal ROS and observed the resulting AMP responses. We first overexpressed DUOX in gut by crossing a *UAS-Duox* transgenic line with *NP1-Gal4* to increase ROS production. Indeed, the global AMP responses were induced in the fat body of uninfected larvae (Figure 1D). We next attempted to feed the larvae a ROS-producing compound, H_2O_2, as a dietary source of ROS. In the larvae fed H_2O_2, neither *Drs-lacZ* nor *Dpt-lacZ* expressions were notably induced in the gut (Figure 1E), suggesting that stimulation of intestinal ROS alone, without infection, did not induce local AMP response in gut. However, the ingestion of H_2O_2 markedly increased the expressions of *Drs-lacZ* and *Dpt-lacZ* in fat body, closely resembling the same response in the fat body when ingested *Ecc15* (Figure 1E). These results suggested that intestinal ROS did not induce an AMP response in the local organ, but in a remote organ. To exclude the possibility of a direct catalytic effect of H_2O_2 on immune regulatory functions, we fed larvae dextran sodium sulfate (DSS), which has been used to induce colitis and intestinal ROS production in a mouse model (Seril et al., 2003). As was found with H_2O_2, the ROS level and *Drs-GFP* reporter expression were both increased (Figure 1F). Moreover, the endogenous *Drs* and *Dpt* levels in fat body were also increased by DSS, but this activation was suppressed by NAC (Figure 1G). These findings provide compelling evidence that intestinal oxidative stress, when stimulated by infection, chemicals, or genetic means, plays an essential role in initiating a gut-to-fat body immunological communication.

Rel Is Essential for Intestinal ROS-Induced Global AMP Responses

Previous studies revealed that Rel is pivotal for global AMP expressions when larvae ingested *Ecc15* (Lemaitre and Hoffmann, 2007). To test whether the global AMP responses in the fat body to intestinal ROS stress were also regulated by the Rel activity, we fed *Rel* null mutant (Rel^{E20}) larvae with H_2O_2 and determined the AMP responses. The global AMP responses were found to be suppressed by the disruption of Rel function (Figure 2A). Furthermore, we investigated whether the specific depletion of *Rel* in fat body (*r4-Gal4 > UAS-relish-RNAi*) or in gut (*NP1-Gal4 > UAS-relish-RNAi*) also reduced the AMP responses. Results suggested that the global AMP expressions in fat body of larvae fed H_2O_2 were diminished upon depletion of *Rel* in fat body, but not in gut (Figure 2B). We also noted that Rel, not Dorsal, was translocalized into the nucleus of fat body after feeding larvae H_2O_2 (Figures 2C), suggesting that only Rel was activated in the fat body. Therefore, Rel may act genetically as a dominant regulator of intestinal ROS-induced global AMP responses in fat body.

Intestinal ROS Interplay with NO Signaling

We speculated whether there is a potential link between intestinal ROS and NO signaling because a previous report showed that the induction of NO by ingestion of a NO donor (SNAP) in larvae also triggers AMP responses in fat body (Foley and O'Farrell, 2003). To test this idea, we fed the larvae H_2O_2 and determined whether the expression of NO synthase (NOS) in gut was altered. The *NOS* mRNA level was found elevated in the gut (Figure 3A), prompting a possibility that the ROS-induced NO production might contribute to the global AMP responses in fat body. To distinguish contributions of NO in the ROS-induced AMP responses in fat body, we blocked the NO production by a pharmacological inhibitor of NOS (L-NAME) when H_2O_2 was provided as a dietary source of ROS. Results showed that both the endogenous *Dpt* and *Dpt-lacZ* reporter expression levels were decreased, whereas the same experiments with *Drs* did not show notably altered expression levels (Figures 3B and 3C). These results suggested that intestinal ROS induced NO production in stimulating *Dpt* but not *Drs* expressions in fat body.

Increasing evidence also suggests that NO can induce ROS overproduction (Swindle and Metcalfe, 2007). Thus, we tested if exogenous NO increased intestinal ROS levels. In the larvae exposed to SNAP, intestinal ROS levels were, on the contrary, decreased (Figure S1A), suggesting a negative feedback mechanism of NO on ROS.

Since the intestinal NO signaling also implicates hemocytes as a relay signal that triggers fat body *Dpt* response (Foley and O'Farrell, 2003), we investigated whether hemocytes conveyed the intestinal ROS signaling in inducing AMP responses in fat body. To test this possibility, we fed a *l(3)hem* mutant larvae, in which a mutation blocks hematopoiesis, H_2O_2 and determined the AMP responses in fat body. The results suggested that the *Dpt* levels were obviously decreased. The *Drs* levels were also decreased, but to a lesser extent (Figure 3D). In addition, we fed the *l(3)hem* larvae *Ecc15* and found that the *Dpt*, not *Drs*, expression was significantly decreased compared to wild-type control (Figure S1B). We suggest that hemocytes may function as an important signal relay between intestinal ROS-NO signaling and fat body *Dpt* response to *Ecc15* oral infection. Although intestinal ROS were also required for the *Drs* response, the signaling of NO-hemocyte did not appear to be crucial for this AMP response.

AP-1 Suppresses Rel-Mediated *Drs* Expression, but It Is Diminished by Intestinal ROS

Previous studies have demonstrated that several transcription factors, including AP-1 (a heterodimer of Jra/Kay) and Caudal, can suppress Rel induction of AMP production (Kim et al., 2007; Ryu et al., 2008). Hence, it is possible that the activity of Rel is restricted by these repressors. Because Caudal has already been shown to function specifically in gut, not in fat

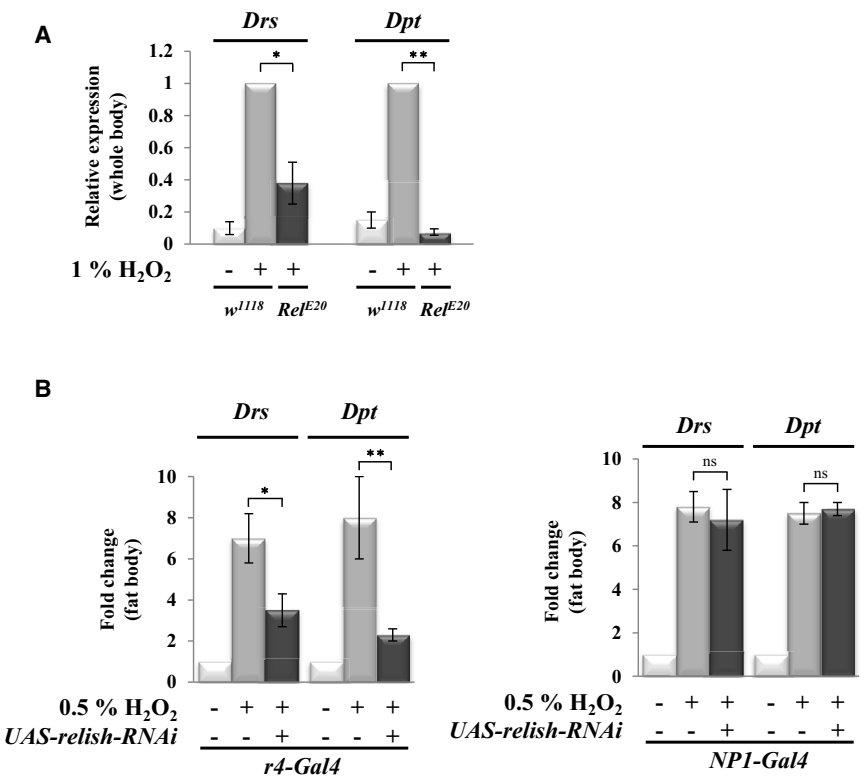

Figure 2. Rel Activation Modulates Global AMP Responses to Intestinal ROS

(A) Disruption of Rel function suppresses the AMP responses to intestinal ROS stress. Shown is qRT-PCR analysis of *Dpt* and *Drs* expressions in the *Rel* null mutant (Rel^{E20}) versus w^{1118} control larvae that ingested 1% H_2O_2 for 6 hr.

(B) Depletion of Rel in fat body suppresses the intestinal ROS-triggered AMP responses. Shown are AMP expressions of the fat body of *r4-GAl4 > UAS-relish-RNAi* or *NP1-Gal4 > UAS-relish-RNAi* versus *Gal4* controls in larvae that ingested 0.5% H_2O_2 for 12 hr.

(C) Double immunostaining for Rel (green) or Dorsal (green) plus nucleus (DAPI, blue) in fat body of w^{1118} larvae that ingested 1% H_2O_2 or PBS for 12 hr. *E. coli* and *M. luteus* were used as positive controls for induction of nucleus translocation for Rel and Dorsal, respectively. Error bars in (A) and (B) represent mean ± SD from three independent experiments. ns indicates no significant change.

body (Ryu et al., 2008), we chose to investigate the repressive role of AP-1. The larvae with Jra overexpression in fat body (*r4-Gal4 > UAS-jra*) were found to have a much lower *Drs* expression in the fat body than control larvae when fed *Ecc15*, whereas the *Dpt* expression was not notably altered (Figure 4A). Conversely, decreasing AP-1 repressor activity by RNAi knockdown of Jra in the fat body (*r4-Gal4 > UAS-jra-RNAi*) further increased *Drs* and *Dpt* expression upon *Ecc15* oral infection (Figure 4B). This inhibitory role of AP-1 in AMP expression was Rel dependent, as evidenced by the decreases in both the *Drs* and *Dpt* expressions in the *jra*-knockdown fat body brought about by reducing one copy of *Rel* ($Rel^{E20/+}$) (Figure 4B). These results suggested that Rel activity was restricted by AP-1 through the suppression of AMP expressions in the fat body.

Hence, we hypothesized that AP-1 activity would be silenced and Rel activity would be resumed when the larvae were fed *Ecc15* or when intestinal DUOX was overexpressed. Although the Jra protein level in gut was increased by the *Ecc15* infection (Figure S2A), its levels in the fat body were decreased. Likewise, when DUOX was overexpressed in gut (*NP1-Gal4 > UAS-Duox*), the Jra level was also decreased in fat body (Figure 4C), suggesting that the repressive role of AP-1 was undermined by the presence of intestinal ROS. Since increasing intestinal ROS also promoted NO production, we investigated whether the induction of NO altered AP-1 activity. Intriguingly, in larvae fed SNAP, the Jra protein level was increased in the fat body (Figure 4D), opposite to that observed by ROS induction. Although the mechanism underlying this is not yet known, we speculate that the ROS-NO interplay may contribute to a tight regulation of the AP-1 activity in the Rel-dependent AMP responses. Together, these findings indicate that the Rel activity in fat body is restricted by AP-1, but such activity is undermined by intestinal ROS stress.

Cytokine Signaling Inhibits Intestinal ROS-Induced AMP Response in Fat Body

Because the *Drosophila* intestinal ROS stress also activates cytokine/JAK/STAT signaling pathway in gut epithelial cell turnover expression (Buchon et al., 2009), we speculated that the cytokine secretion from enterocytes might initiate the immunological response in fat body. To test whether cytokine was induced in the larval gut by *Ecc15* oral infection, we examined *upd3* (*unpaired 3*, a *Drosophila* cytokine) expressions in fat

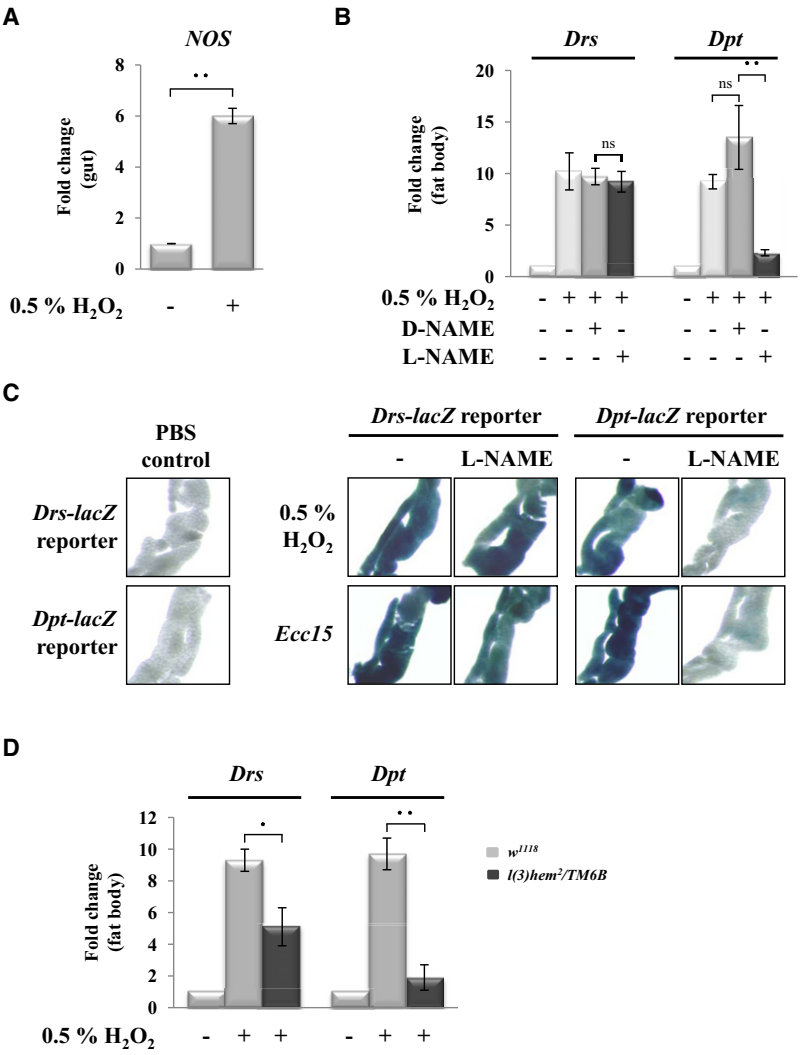

Figure 3. ROS-Induced AMP Responses Mediated by NO and Hemocytes

(A) ROS stress induces elevation of *NOS* mRNA level in gut. The intestinal *NOS* was determined by qRT-PCR after ingestion of 0.5% H_2O_2 for 12 hr.

(B) Inhibition of NO suppresses intestinal ROS-induced *Dpt*, not *Drs*, expression in fat body. Shown is qRT-PCR analysis of AMP expressions in fat body of larvae co-fed 0.5% H_2O_2 with or without 200 mM L-NAME for 12 hr. *Drs* and *Dpt* expressions were not significantly affected by the L-NAME control drug (D-NAME).

(C) Inhibition of NO suppresses intestinal ROS-induced *Dpt-lacZ*, not *Drs-lacZ*, reporter expression in fat body. Shown is β-gal staining of *Dpt-lacZ* or *Drs-lacZ* reporter in the fat body of larvae cofed 0.5% H_2O_2 for 12 hr or *Ecc15* for 24 hr with or without 200 mM L-NAME.

(D) Hemocytes mediate fat body AMP expressions in larvae that ingested H_2O_2. AMP responses in fat body of *l(3)hem*^{2}*/TM6B* larvae compared to w^{1118} fed H_2O_2. Error bars in (A), (B), and (D) represent mean ± SD from three independent experiments. ns indicates no significant change. See also Figure S1.

body and in gut over the time course of infection. Indeed, *upd3* expressions in the gut were notably elevated during the time course of infection but were almost undetectable in the fat body (Figure S2B). We then tested whether interruption of cytokine receptor-mediated signaling disrupted the *Ecc15*-induced AMP response in fat body. By knocking down the only *STAT* (*Stat92E*) gene in the fat body of *Drosophila* via crossing *UAS-Stat92E*^{RNAi} with a fat body-specific Gal4 line (*r4-Gal4*), we examined the fat body AMP responses to *Ecc15* oral infection. Surprisingly, the global AMP responses were further increased, not decreased, in the fat body (Figure 4E), suggesting that cytokine signaling suppressed fat body AMP responses upon *Ecc15* infection. We speculated that for global AMP genes to be fully expressed, the inhibitory function of STAT signaling must be deactivated upon *Ecc15* infection. Our experiments found *STAT-GFP* reporter expression to be suppressed in the fat body, but not in the gut (Figure 4F). To confirm this, we investigated whether *Socs36E*, a JAK/STAT signaling target, was also suppressed by the infection. Indeed, the results showed that the *Socs36E* expression in fat body was decreased during the time course of infection by *Ecc15* (Figure 4G). These findings show that cytokine/STAT signaling inhibits *Ecc15* infection-mediated AMP responses in the fat body.

DISCUSSION

This study demonstrates a unique signaling profile underlying the importance of oxidative stress in an organ to the activation of systemic immune responses in remote organs. Here we propose a model for the immunological communication between gut and fat body. Intestinal ROS stress, which is induced by *Ecc15* oral infection, can trigger the Rel transcription activity for global AMP expressions in fat body through either NO-dependent or NO-independent pathways. ROS signaling triggers transcription of NOS in gut, which then promotes the production of NO and activation of the IMD pathway to express *Dpt* in fat body. Hemocytes may function as a signal-relaying organ between the gut and fat body for this *Dpt* response. However, the activation of *Drs* transcription appears not to be mediated via this ROS-NO signaling pathway but directly through ROS (Figure 4H). Interestingly, for the ROS-induced *Drs* expression, there might be alternative pathways involved, because the depletion of Rel only partially decreased the *Drs* expression (Figures 2A and 2B).

It is important to note that our study showed a homeostatic interplay between intestinal ROS and NO in regulating the Rel-dependent *Dpt* response to *Ecc15* oral infection. Although the *Drs* response was also dependent on intestinal ROS, the NO-hemocyte signaling did not appear to be involved. This conclusion is supported by a previous report that a NO-independent pathway is pivotal for the *Drs* response to *Ecc15* oral infection (Foley and O'Farrell, 2003).

We also found that the Rel activity in fat body was restricted by AP-1, but this repressive activity was substantially suppressed

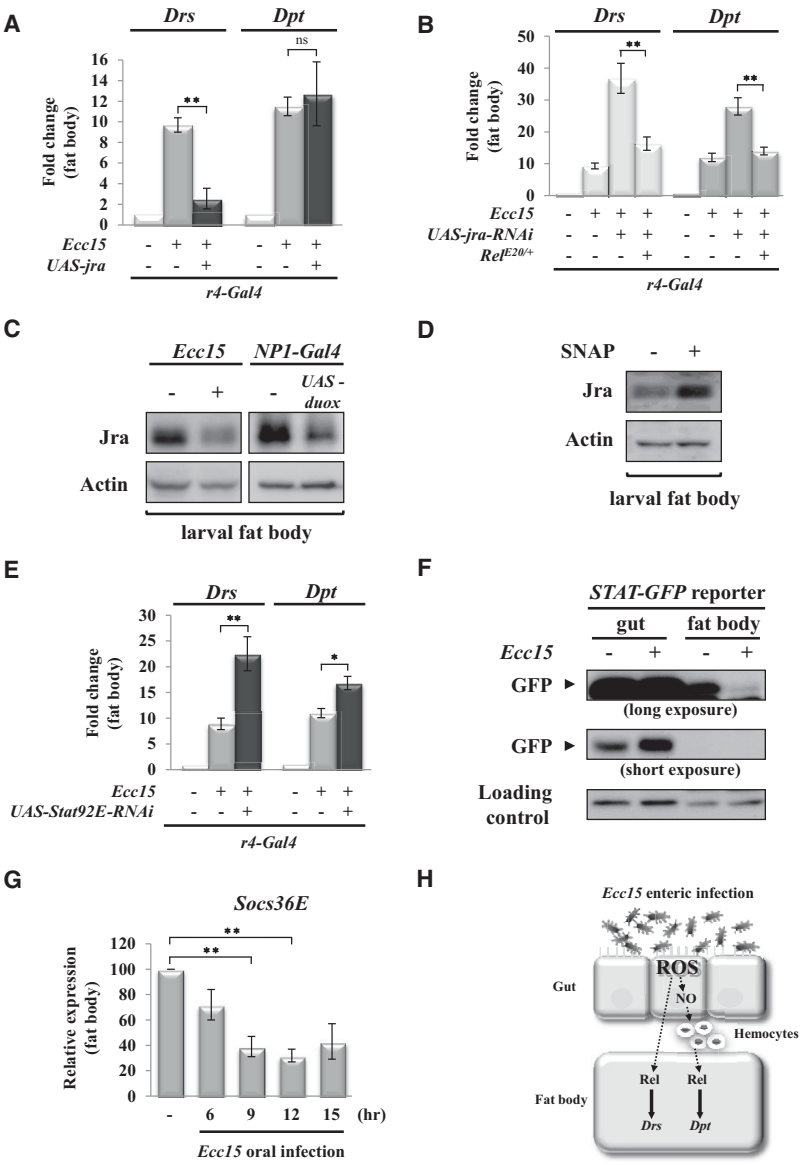

Figure 4. AP-1 Represses Rel-Dependent AMP Expressions, but It Is Undermined by Intestinal ROS

(A) Increasing AP-1 activity in the fat body suppresses *Drs*, not *Dpt*, expression in that organ. Shown is qRT-PCR analysis of *Drs* and *Dpt* expressions in the fat body of *r4-Gal4* > *UAS-jra* larvae versus *r4-Gal4* control fed *Ecc15* for 24 hr.

(B) Decreasing AP-1 repressor activity enhances Rel-dependent *Drs* and *Dpt* expressions. RNAi knockdown of Jra in the *r4-Gal4* > *UAS-jra-RNAi* fat body increased the *Ecc15*-induced *Drs* and *Dpt* expressions. The *Ecc15*-induced AMP expressions in the *jra* knockdown fat body were diminished by reducing one copy of *Rel* ($Rel^{E20/+}$).

(C and D) Intestinal ROS decrease AP-1 protein in the fat body, whereas NO increases it. Western blot analysis of Jra in total lysates of *NP1-Gal4* > *UAS-Duox* fat body (C) or w^{1118} fat body of larvae fed *Ecc15* for 24 hr (C) or 15 mM SNAP for 6 hr (D).

(E) Depletion of Stat92E in fat body increases the *Ecc15*-induced AMP responses. Shown is qRT-PCR analysis of *Drs* and *Dpt* expressions in the fat body of *r4-Gal4* > *UAS-Stat92E*^{RNAi} transgenic larvae versus *r4-Gal4* control fed *Ecc15* for 24 hr.

(F and G) STAT signaling in fat body is inactivated in larvae fed *Ecc15*. Western blot analysis of *STAT-GFP* reporter expression in the gut and in the fat body of larvae (F). Shown is qRT-PCR analysis of *Socs36E*, a targeted gene of Stat92E, in the fat body of larvae (G).

(H) A signaling model for the intestinal ROS-induced immunological communication between organs. The model predicts that intestinal ROS, which can be induced by *Ecc15* oral infection, can trigger the Rel transcription activity for global AMP expressions in fat body through NO-dependent or NO-independent pathways. Hemocytes may function as a signal relay module between gut and fat body for the *Dpt* response. Error bars in (A), (B), (E), and (G) represent mean ± SD from three independent experiments. ns indicates no significant change. See also Figure S2.

by intestinal ROS stress. Interestingly, although AP-1 and cytokine/STAT signaling pathways were both activated in the gut by enteric infection, they were concurrently suppressed in the fat body, suggesting that some of the regulatory molecules involved in interorgan immunological communication might be oppositely regulated in the gut and the fat body. Also of great interest is the fact that cytokine signaling was found to act negatively in mediating the fat body AMP response to *Ecc15* oral infection. This leads to the question: if it is not cytokines that act as positive extracellular messengers in initiating interorgan communication, what signaling molecules are released in the circulation that can have this effect? Some ROS, such as H_2O_2, are emerging as important intracellular second messengers in various signalings, but because they degrade rapidly, they are considered to be incapable of acting as extracellular messengers in the modulation of interorgan signaling (Lee, 2008). Circulating hemocytes directed to the infection site may act as a signaling device, as suggested by other studies (Basset et al., 2000; Foley and O'Farrell, 2003). Another possibility is that host molecules, such as endogenous DNA and host ligands, which can be released at a wound site, may play a role in inducing AMP expression (Lemaitre and Hoffmann, 2007).

There is a current growing body of evidence identifying two crucial roles of intestinal ROS in modulating *Drosophila* immunity in the gut, one involving in microbicidal activity and the other involving stem cell renewal (Buchon et al., 2009; Ha et al., 2005). This current study suggests that, in addition to these roles, ROS may play a role in facilitating organ-to-organ immunological communication. Whether such a mechanism has been conserved in mammals is currently under investigation.

EXPERIMENTAL PROCEDURES

Fly Stocks

Dpt-lacZ (Basset et al., 2000), *Drs-lacZ* (Basset et al., 2000), *Drs-GFP* (Zaidman-Rémy et al., 2006), and *10xSTAT-GFP* (Bach et al., 2007) reporter lines, Rel^{E20} and $l(3)hem^2/TM6B$ mutants, and w^{1118} were used in this study. UAS-based approach was used to overexpress or knock down *Duox*, *jra*, *relish*,

and *Stat92E* driven by *r4-Gal4* (Lee and Park, 2004) or *NP1-Gal4* (Zaidman-Rémy et al., 2006) lines.

Larval Oral Infection and Real-Time Quantitative PCR Analysis

Ecc15 oral infection of larvae was performed as described previously (Basset et al., 2000). Early foraging 3rd instar larvae (65–68 hr at 25°C after hatching) were used for this assay. Larvae orally ingested concentrated *Ecc15* (OD_{100}) cofed with or without 100 mM NAC (Sigma) that were mixed in standard *Drosophila* food (1 ml *Ecc15* or 1 ml *Ecc15* + NAC solution mixed in 4 g food). Fat bodies or guts were collected after 24 hr of *Ecc15* oral infection or 12 hr of H_2O_2 ingestion and used for analyzing AMP response by qRT-PCR and β-gal staining. The following primers were used in qRT-PCR analysis: *Dpt* forward 5′-GTTCACCATTGCCGTCGCCTTAC-3′, *Dpt* reverse 5′-CCCAA GTGC TGTCCATATCCTCC-3′; *Drs* forward 5′-TTGTTCGCCCTCTTCGCTGT CCT-3′, *Drs* reverse 5′-GCATCCTTCGCACCAGCACTTCA-3′; *rp49* forward 5′-AGATCGTGA AGAAGCGCACCAAG-3′, *rp49* reverse 5′-CACCAGGAACT TCTTGAATCCGG-3′; *NOS* forward 5′-CCGCACGACAA AATACC-3′, *NOS* reverse 5′-GCGTTAGTTGGGCAAG-3′; *Socs36E* forward 5′- AAGTGCACACT GTCGAATGG-3′, and *Socs36E* reverse 5′-TTCCCCGTTTTC ACGTTATC-3′.

Antibody Preparation

Rabbit polyclonal anti-Rel antibody was raised against the N-terminal fragment (amino acid 270–545) of Rel and affinity purified.

Oxidative Stress Stimuli

Standard food containing H_2O_2, DSS, or *Ecc15* was used for larval oral ingestion. Intestinal ROS levels were analyzed after *Ecc15* or DSS ingestion for 6 or 24 hr, respectively. For L-NAME treatment, larval guts were used for analyzing *NOS* mRNA level and fat bodies were utilized for analyzing AMP expressions after coingestion H_2O_2 or *Ecc15* with or without 200 mM L-NAME (Sigma). Fat bodies of *l(3)hem*^{2}*/TM6B* larvae that ingested H_2O_2 were used for analyzing AMP expression levels compared to w^{1118}.

ROS Measurement

ROS level in the larval gut was analyzed by 10 μM DCF-DA fluorescent dye (Sigma), which was added into the standard food-medium of *Drosophila* for the cultivation. The DCF-DA fluorescent signal was analyzed by fluorescence microscopy at 490 nm excitation and 525 nm emissions.

Immunostaining and Immunoblotting

Immunostaining of the larval fat body was performed according to previous description (Lin et al., 2009). After fixation and blocking steps, fat bodies were incubated with anti-N-Rel or anti-Dorsal antibodies (Developmental Studies Hybridoma Bank, DSHB) and counterstained with cell nucleus dye DAPI. FITC-conjugated secondary antibody was purchased from Jackson Laboratories. Confocal images were obtained using Leica TCS SP5 confocal microscope. For immunoblotting analysis, fat bodies or guts were collected from w^{1118}, *NP1-Gal4* > *UAS-Duox*, or *10xSTAT-GFP* reporter larvae that ingested *Ecc15*, 15 mM SNAP (Sigma), or PBS as control. Tissue total lysates were separated by SDS-PAGE, blotted, and probed with anti-GFP (1:5,000, BD living color) or anti-Jra (1:5,000, Santa Cruz), according to standard procedure.

Statistics

Student's t test was used for two-group comparisons. The *p value < 0.05 was considered significant and **p value < 0.01 highly significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at doi:10.1016/j.chom.2012.03.004.

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REFERENCES

Bach, E.A., Ekas, L.A., Ayala-Camargo, A., Flaherty, M.S., Lee, H., Perrimon, N., and Baeg, G.H. (2007). GFP reporters detect the activation of the Drosophila JAK/STAT pathway in vivo. Gene Expr. Patterns *7*, 323–331.

Basset, A., Khush, R.S., Braun, A., Gardan, L., Boccard, F., Hoffmann, J.A., and Lemaitre, B. (2000). The phytopathogenic bacteria Erwinia carotovora infects Drosophila and activates an immune response. Proc. Natl. Acad. Sci. USA *97*, 3376–3381.

Buchon, N., Broderick, N.A., Chakrabarti, S., and Lemaitre, B. (2009). Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila. Genes Dev. *23*, 2333–2344.

Foley, E., and O'Farrell, P.H. (2003). Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in Drosophila. Genes Dev. *17*, 115–125.

Ha, E.M., Oh, C.T., Bae, Y.S., and Lee, W.J. (2005). A direct role for dual oxidase in Drosophila gut immunity. Science *310*, 847–850.

Ha, E.M., Lee, K.A., Seo, Y.Y., Kim, S.H., Lim, J.H., Oh, B.H., Kim, J., and Lee, W.J. (2009). Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in drosophila gut. Nat. Immunol. *10*, 949–957.

Han, Z.S., and Ip, Y.T. (1999). Interaction and specificity of Rel-related proteins in regulating Drosophila immunity gene expression. J. Biol. Chem. *274*, 21355–21361.

Hedengren, M., Asling, B., Dushay, M.S., Ando, I., Ekengren, S., Wihlborg, M., and Hultmark, D. (1999). Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. Mol. Cell *4*, 827–837.

Karpac, J., Younger, A., and Jasper, H. (2011). Dynamic coordination of innate immune signaling and insulin signaling regulates systemic responses to localized DNA damage. Dev. Cell *20*, 841–854.

Kim, L.K., Choi, U.Y., Cho, H.S., Lee, J.S., Lee, W.B., Kim, J., Jeong, K., Shim, J., Kim-Ha, J., and Kim, Y.J. (2007). Down-regulation of NF-kappaB target genes by the AP-1 and STAT complex during the innate immune response in Drosophila. PLoS Biol. *5*, e238.

Lee, W.J. (2008). Bacterial-modulated signaling pathways in gut homeostasis. Sci. Signal. *1*, pe24.

Lee, G., and Park, J.H. (2004). Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in Drosophila melanogaster. Genetics *167*, 311–323.

Lemaitre, B., and Hoffmann, J. (2007). The host defense of Drosophila melanogaster. Annu. Rev. Immunol. *25*, 697–743.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973–983.

Lin, T.Y., Huang, C.H., Kao, H.H., Liou, G.G., Yeh, S.R., Cheng, C.M., Chen, M.H., Pan, R.L., and Juang, J.L. (2009). Abi plays an opposing role to Abl in Drosophila axonogenesis and synaptogenesis. Development *136*, 3099–3107.

Macdonald, T.T., and Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. Science *307*, 1920–1925.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature *388*, 394–397.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., et al. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085–2088.

Rezaie, A., Parker, R.D., and Abdollahi, M. (2007). Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? Dig. Dis. Sci. *52*, 2015–2021.

Rock, K.L., Latz, E., Ontiveros, F., and Kono, H. (2010). The sterile inflammatory response. Annu. Rev. Immunol. *28*, 321–342.

Rosetto, M., Engström, Y., Baldari, C.T., Telford, J.L., and Hultmark, D. (1995). Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a Drosophila hemocyte cell line. Biochem. Biophys. Res. Commun. *209*, 111–116.

Ryu, J.H., Kim, S.H., Lee, H.Y., Bai, J.Y., Nam, Y.D., Bae, J.W., Lee, D.G., Shin, S.C., Ha, E.M., and Lee, W.J. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. Science *319*, 777–782.

Seril, D.N., Liao, J., Yang, G.Y., and Yang, C.S. (2003). Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. Carcinogenesis *24*, 353–362.

Swindle, E.J., and Metcalfe, D.D. (2007). The role of reactive oxygen species and nitric oxide in mast cell-dependent inflammatory processes. Immunol. Rev. *217*, 186–205.

Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J.M., Lemaitre, B., Hoffmann, J.A., and Imler, J.L. (2000). Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity *13*, 737–748.

Vidal, S., Khush, R.S., Leulier, F., Tzou, P., Nakamura, M., and Lemaitre, B. (2001). 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. *15*, 1900–1912.

Zaidman-Rémy, A., Hervé, M., Poidevin, M., Pili-Floury, S., Kim, M.S., Blanot, D., Oh, B.H., Ueda, R., Mengin-Lecreulx, D., and Lemaitre, B. (2006). The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection. Immunity *24*, 463–473.