Transcriptional up-regulation of the TGF-β intracellular signaling transducer Mad of Drosophila larvae in response to parasitic nematode infection

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Abstract
The common fruit fly Drosophila melanogaster is an exceptional model for dissecting innate immunity. However, our knowledge on responses to parasitic nematode infections still lags behind. Recent studies have demonstrated that the well-conserved TGF-β signaling pathway participates in immune processes of the fly, including the anti-nematode response. To elucidate the molecular basis of TGF-β anti-nematode activity, we performed a transcript level analysis of different TGF-β signaling components following infection of D. melanogaster larvae with the nematode parasite Heterorhabditis gerrardi. We found no significant changes in the transcript level of most extracellular ligands in both bone morphogenic protein (BMP) and activin branches of the TGF-β signaling pathway between nematode-infected larvae and uninfected controls. However, extracellular ligand, Scw, and Type I receptor, Sax, in the BMP pathway as well as the Type I receptor, Babo, in the activin pathway were substantially up-regulated following H. gerrardi infection. Our results suggest that receptor up-regulation leads to transcriptional up-regulation of the intracellular component Mad in response to H. gerrardi following changes in gene expression of intracellular receptors of both TGF-β signaling branches. These findings identify the involvement of certain TGF-β signaling pathway components in the immune signal transduction of D. melanogaster larvae against parasitic nematodes.

Keywords
Drosophila, Heterorhabditis, immunity, parasitism, TGF-β signaling

Introduction
The ability of parasitic nematodes to infect a range of invertebrate and vertebrate hosts poses a serious threat to global health and agriculture and carries major socio-economic consequences. Furthermore, we are currently lacking a good model system for studying the molecular basis of anti-parasitic immune responses, which limits our understanding of the mechanisms underlying these host–parasite interactions.1,2

The common fruit fly, Drosophila melanogaster, has been used extensively as an excellent model for innate immune processes, from analyzing immune signal transduction to characterizing immune function regulation. Identification of the conserved NF-κB signaling pathways Toll and immune deficiency pathway (IMD) has demonstrated that D. melanogaster is able to discriminate between different classes of pathogens and activate a wide range of responses.3−7 Other conserved signaling pathways, such as the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and c-Jun N-terminal kinase (JNK), also participate in immune reactions.8−10 The well-conserved TGF-β signaling pathway, which is involved in inflammation and tissue repair in mammals, has been shown...
previously to be involved in the immune response to wounding and bacterial infection in D. melanogaster.\(^{11,12}\) This is achieved through NF-κB regulation of decapentaplegic (dpp) and dawdle (daw). Wounding activates dpp and represses the production of antimicrobial peptides, whereas daw limits infection-induced melanization. In addition, our recent studies have demonstrated the participation of TGF-β signaling in the anti-nematode immune response in adult flies.\(^{13}\) Our results revealed that the extracellular ligands dpp and daw are transcriptionally induced following nematode infection, and they also modulate the survival ability of flies against these parasites.

The TGF-β pathway is composed of two signaling branches: the bone morphogenic protein (BMP) and the activin pathways. TGF-β pathway in D. melanogaster consists of extracellular ligands that bind to type I and type II receptors, intracellular signal transducers and nuclear read-out genes.\(^{14,15}\) Extracellular ligands of the BMP pathway decapentaplegic (Dpp), glass bottom boat (Gbb) and screw (Scw) bind to type I receptors saxophone (Sax) and thick veins (Tkv), and type II receptors punt (Put) or wishful thinking (Wit). Receptor binding leads to signal transduction that is mediated by Smad proteins, more specifically in D. melanogaster, mothers against dpp (Mad). Similarly, extracellular ligands of the activin pathway activinβ (Actβ), dawdle (Daw) and myoglianin (Myo) bind to type I receptor, baboon (Babo) and type II receptors punt or wit. Signal transduction is mediated by intracellular protein, Smad on X (Smox).\(^{16,17}\) Interestingly, Mad activation can be achieved via signaling through receptor Babo.\(^{18}\)

Previous studies in D. melanogaster have demonstrated that parasitic nematodes of the genus Heterorhabditis are able to infect and kill adult flies and larvae,\(^{19–27}\) which leads to transcriptional up-regulation of genes in Toll, IMD, JAK/STAT and TGF-β signaling pathways.\(^{21,25,26}\) Here, we investigated the regulation of TGF-β signaling pathway upon infection of D. melanogaster larvae with the nematode parasite Heterorhabditis gerrardi. This parasitic nematode harbors the mutualistic bacteria Photobacterium asymbiotica, which can act as insect and human pathogen.\(^{29–31}\) Upon infection, the bacteria are expelled from gut of nematode infective juveniles into the hemolymph of the insect host, where they multiply and secrete a cocktail of toxins and virulence factors that promote insect death and therefore provide a favorable environment for H. gerrardi development.\(^{12,13}\)

Using a real-time quantitative (q)RT-PCR approach to detect and reliably measure the transcript levels of genes encoding various TGF-β signaling components, here we demonstrate that the intracellular signaling transducer Mad is up-regulated in D. melanogaster larvae upon infection with H. gerrardi nematodes. This is achieved through both signaling branches of the TGF-β signaling pathway, BMP and activin. The reported findings integrate our understanding of the transcriptional regulation of certain TGF-β superfamily members in the Drosophila immune signaling during infection with potent parasitic nematodes. Further studies into the specific function of these signaling components in response to parasitic nematodes could lead to better understanding of the mechanisms that underlie host–parasite interactions.

### Materials and methods

#### Fly and nematode stocks

All stocks were raised on standard cornmeal-soy based food (Cat. No. 101-NV, Meidi laboratories) with a few granules of dry baker’s yeast at 25°C, 12:12 light:dark photoperiod and 60% humidity. A fly strain carrying P-bac insertion Pbac(Pr)/Mad\(^{60574}\) was obtained from Exelixis Harvard Medical School. Strain w\(^{1118}\) was used as background control in all experiments and it was also obtained from the Bloomington Drosophila Stock center.

Parasitic nematodes used in the experiments were H. gerrardi, amplified in the fourth instar larvae of the wax moth Galleria mellonella using the water trap technique.\(^{34}\) H. gerrardi infective juveniles used in experiments were 1–5 wk old.

#### Nematode infection

For infection of D. melanogaster with the H. gerrardi infective juveniles, second and third instar larvae were collected and rinsed briefly in water and then placed in wells of a 96-well plate (one larva per well), each containing 100μl of 1.25% agarose. Infective juveniles were washed and adjusted to the final density of approximately 100 per larva, and 10μl of nematode suspension was added to a single D. melanogaster larva. For uninfected controls, 10μl of sterile water was added to each larva. The 96-well plate was covered with plastic film, which was punctured to provide aeration. The plates were kept in dark and survival was quantified under a stereo-microscope. Survival rates in response to H. gerrardi nematode infection were determined twice per d for 72 h based on larval movement.

#### Gene transcript analysis

To analyze the transcriptional regulation of TGF-β signaling components in D. melanogaster responding to H. gerrardi nematodes, larvae were infected with infective juveniles as previously described and subsequently collected at 24, 40, and 64 h representing an early, an intermediate, and a late time point during infection, respectively. For each experiment, 20 second or early third instar larvae were infected, and four to five live
individuals per replicate were collected at specified time points. Larvae of the same developmental stages were treated with water only and acted as uninfected controls. Total RNA was extracted from whole larvae using TRIzol Reagent (Ambion, Life Technologies). Reverse transcription was performed using iScript™ cDNA Synthesis Kit (Bio-Rad). iTaq™ Universal SYBR® Green Supermix (Bio-Rad) was used for qRT-PCR using CFX96™ Real-Time System, C1000™ Thermal Cycler with the following conditions: 95°C for 2 min, 40 cycles of 95°C for 15 s and 61°C for 30 s, 95°C for 15 s, 65°C for 5 s and 95°C for 5 s. CFX Manager 3.1 (Bio-Rad) was used for data analysis. Primers used to quantify mRNA levels are listed in Table 1.

**Statistical analysis**

GraphPad Prism (v7.0 c) was used for data plotting and statistical analyses. Three independent survival experiments were performed, and the results were analyzed with Log-rank (Mantel-Cox) test. Experiments for TGF-β gene transcript levels were repeated three times, and the results were processed with unpaired t-test.

**Results**

**H. gerrardi infection affects the expression of extracellular ligand scw in the BMP pathway of D. melanogaster**

To investigate the regulation of TGF-β signaling in D. melanogaster larvae in response to infection with parasitic nematode H. gerrardi, we first analyzed the induction of the extracellular ligands scw, dpp, and gbb in the BMP pathway (Figure 1). While there was no significant difference in the transcript levels of dpp between infections with H. gerrardi infective juveniles and uninfected controls at any of the time points (Figure 1a,b), we found significantly higher transcript levels of scw at the mid timepoint (Figure 1c, 40 h post infection) following nematode infection. These results indicate that specific extracellular ligands in the BMP branch of the TGF-β signaling pathway are substantially up-regulated in D. melanogaster larvae following parasitic nematode infection.

**mad is transcriptionally up-regulated via type I receptor Sax of the BMP pathway**

Following up the results above, we also assessed potential changes in the expression of intracellular components of the BMP pathway upon H. gerrardi challenge. We found that the intracellular receptor sax was transcriptionally up-regulated at the mid timepoint (40 h) in response to H. gerrardi and sequentially decreased at 64 h (Figure 1d). Interestingly, the transcript level pattern of mad, which is responsible for mediating the signal transduction in the BMP branch of the TGF-β pathway, was significantly up-regulated at 40 h post nematode infection compared with uninfected controls, and this up-regulation was substantially higher than the up-regulation observed for scw or sax (Figure 1e). Together, these results suggest that mad regulation is directionally achieved through the regulation of type I receptor sax during infection of D. melanogaster larvae with H. gerrardi nematode parasites.

**H. gerrardi infection does not affect the expression of extracellular ligands in the activin pathway of D. melanogaster**

We then examined the regulation of the extracellular ligands actβ, myo, mav, and daw in the activin branch of the TGF-β signaling pathway following infection of

| Gene name | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|-----------|------------------------|-----------------------|
| rp49      | GATGACCATCCGCCCAGCA    | CGGACACGACAGCTGCTTGGC |
| diptericin| GCTGCGCATATCGCTTCTACT  | TGTTGAGGTTGGCCCTTCA   |
| actβ      | CCATTCAAAGGCAGACGTTG  | AGCGGGTTGTTGAAAATGCT  |
| babo      | CGCTCCATCCTGTTGATAAGCA | TCTGGTCCTCTGCTTGGGC   |
| daw       | CAGAGGAGCAGATGTACCAGAT| GTGCTGCTCTCCGGTCAAAT  |
| dpp       | TGGGCACCTTTTCAAACGATGT | CAGCGGAATATGAGCCGCGAA |
| gbb       | GGACCTCGGAATGTCTTCTGC  | CTGTTGCTATGTAATCCCCGC |
| mad       | GAGCAAGACGGAGAAGGTTGGG | TAGATACATGCCGCCAGACC  |
| myo       | ATGCTGCGGTTGAGAAAATA   | CGTGCAATATCGAGTTACAGGG|
| sax       | ACCCACCACCCGAGGATGG    | CTCCCTGGATTGCGTTTACT  |
| smox      | GAGCTATCAACACGCAACAGC  | TGCCACACTAAAGCACACTC  |
| scw       | GCATCTGGGGCTGCTTGAAT   | ACCGAGCAGGATCGTCAAA   |
D. melanogaster larvae with H. gerrardi nematodes (Figure 2). We found that none of the extracellular ligands exhibited significant changes in transcript levels compared with the background control strain w1118 at any timepoints post nematode infection (Figure 2a–d). These results suggest that TGF-β signaling in D. melanogaster larvae is not regulated at the level of extracellular ligand up-regulation of the activin pathway following H. gerrardi nematode infection.

Even though the extracellular ligands in the activin branch of the TGF-β signaling pathway were not transcriptionally up-regulated in response to parasitic nematode infection, we included the expression analysis of the receptor babo for its potential to activate mad.18 Indeed, we found that babo was transcriptionally up-regulated in w1118 larvae at the mid timepoint (40 h) following infection with H. gerrardi compared with

D. melanogaster larvae with H. gerrardi nematodes (Figure 2). We found that none of the extracellular ligands exhibited significant changes in transcript levels compared with the background control strain w1118 at any timepoints post nematode infection (Figure 2a–d). These results suggest that TGF-β signaling in D. melanogaster larvae is not regulated at the level of extracellular ligand up-regulation of the activin pathway following H. gerrardi nematode infection.

**Figure 1.** Within BMP signaling pathway, infection of D. melanogaster larvae with H. gerrardi nematodes leads to increased transcript levels of extracellular ligand scw, type I receptor sax, and transcription factor mad. Expression levels of extracellular ligands (a) dpp and (b) gbb are not significantly different from uninfected background control w1118 larvae at 24, 40, and 64 h post infection. (c) Transcript levels of scw are significantly up-regulated at 24 and 40 h post infection compared with uninfected controls (*P = 0.0132 and **P = 0.0038, respectively). (d) Transcript levels of sax are up-regulated at 24 and 40 h post infection compared with uninfected controls (*P = 0.0244 and *P = 0.0399, respectively), followed by a decrease at 64 h post infection (*P = 0.0306). (e) Transcript levels of mad are up-regulated at 24 and 40 h post infection compared with uninfected controls (**P = 0.0048 and ****P < 0.0001, respectively), followed by a decrease at 64 h post infection (**P = 0.0067). Red dotted line at 1 indicates normalization of fold change relative to uninfected controls.
uninfected controls (Figure 2e). In contrast, expression of intracellular protein \textit{smox} downstream of \textit{babo} was not affected by \textit{H. gerrardi} nematode infection (Figure 2f). Collectively, these results suggest that the transcriptional changes of TGF-\(\beta\) signaling in \textit{D. melanogaster} larvae upon \textit{H. gerrardi} nematode infection are regulated via signal transducer Mad, through either of the signaling branches, the BMP pathway, or the activin pathway.

\textbf{mad up-regulation does not contribute to survival and antimicrobial peptide levels in \textit{D. melanogaster} following infection with \textit{H. gerrardi}}

Generally, the extent of Mad involvement in response against nematode infection is still unclear as the survival of \textit{D. melanogaster mad} mutant larvae upon \textit{H. gerrardi} nematode infection was not affected compared with their background control strain \textit{w1118} (Figure 3a). Furthermore, they only marginally triggered immune response, as read-out \textit{dipericin} transcript levels were slightly higher, albeit insignificant, compared with \textit{w1118} (Figure 3b).

Our observation that transcript levels of \textit{scw}, \textit{sax}, and \textit{mad} being up-regulated following infection with \textit{H. gerrardi} nematodes at a mid timepoint (40 h post infection) is in accordance to the activation of Sax by Scw binding that leads to the phosphorylation of intracellular protein Mad. \(^{35}\) Altogether, these results suggest that the transcriptional changes of TGF-\(\beta\) signaling in \textit{D. melanogaster} larvae upon \textit{H. gerrardi} attack are regulated via signal transducer Mad, through either of
the signaling branches, the BMP or the activin pathway (Figure 4), but these molecular events do not provide a survival advantage to survival in response to nematode infection.

Discussion

Parasitic nematodes cause infectious diseases that represent one of the major threats to human health. To understand the molecular mechanisms that regulate host-nematode interaction, it is crucial to develop and exploit tractable research tools.1,2 Previous transcriptomic studies have demonstrated that the insect pathogenic nematodes *H. bacteriophora* are able to infect and kill *D. melanogaster* larvae, and that different types of signaling pathways are induced in *D. melanogaster* following infection with these parasites.25,28 Here, we have examined the molecular regulation of the evolutionarily-conserved TGF-β signaling pathway in *D. melanogaster* larvae upon infection with a potent nematode parasite. TGF-β signaling has a role in tissue repair and inflammation in mammals and is also involved in the anti-pathogen immune response of adult flies.11,13

In this study, we analyzed the transcriptional induction of different *D. melanogaster* BMP and activin signaling components including the type I receptors Sax and Babo upon infection with the parasitic nematode *H. gerrardi*. TGF-β signaling can be regulated in three distinct settings, the extracellular space, the cell membrane, and the intracellular region. At the level of extracellular ligands, we only observed the up-regulation of *scw*, a ligand in the BMP branch. In contrast, expression of *dpp* and *daw*, shown previously to be induced upon nematode infection of *D. melanogaster* adult flies,13 was not altered in larvae compared with uninfected controls, suggesting that the up-regulation of a specific TGF-β ligand, *scw* in this case, is restricted to developmental stage. When *Scw* binds to the type I receptor Sax, it leads to the activation of Mad.
Indeed, we observed increased expression of sax and mad following infection with the nematode parasite H. gerrardi.

In Drosophila S2 cells, Mad up-regulation through Babo has been linked to Daw ligand binding.36 Even though we did not observe up-regulation of extracellular ligands in the activin branch, the type I receptor Babo is up-regulated at a late timepoint following nematode infection and can also lead to activation of Mad.18 These results support the notion that transcriptional induction of mad in D. melanogaster larvae following infection with H. gerrardi parasitic nematodes can be achieved via either the BMP or activin branch (Figure 4). In mammalian hosts, helminth parasite infection triggers the activation of Mad and leads to increase in TGF-β levels.37

The observation that there is no apparent change in transcript levels of other extracellular ligands following infection with H. gerrardi nematodes suggests that the up-regulation of extracellular ligand scw, as well as Type I receptors sax and babo, is nematode-species-specific. Indeed, transcriptional regulation of other extracellular ligands, such as dpp and daw, has been observed following infection with the related parasitic nematode species, H. bacteriophora.13 However, these results were obtained in nematode-infected adult flies. Therefore, our results indicate that the transcriptional changes of TGF-β signaling components through binding of different extracellular ligands can be achieved by different types of parasitic nematode infection in different life stages of D. melanogaster. Furthermore, the activation of a known antimicrobial immune signaling pathway, such as IMD, was not impaired following nematode infection of mad inactivated mutants, which showed similar sensitivity to H. gerrardi infection compared with background controls. These findings imply that, despite the observed differential regulation of mad, there is lack of cross-talk between the expression of this TGF-β signaling component and IMD pathway activation in D. melanogaster larvae in the context of H. gerrardi challenge, and these effects fail to alter insect survival against these nematodes.

Our current results follow up on previous findings and establish the involvement of TGF-β pathway in modulating insect-nematode molecular interactions. Our finding that the TGF-β intracellular signaling transducer mad can be up-regulated by both branches of the TGF-β signaling pathway (BMP and activin) indicates a potentially key role in the Drosophila signaling response to H. gerrardi. Further research involving related entomopathogenic nematodes, such as H. bacteriophora and H. downesi, or nematodes from the genus Steinernema together with natural insect hosts, such as lepidopteran larvae, could provide additional insight on whether activation of this pathway is nematode-specific or conserved as a wider insect anti-nematode response, and whether there is interaction with Toll, JAK/STAT, and JNK pathways, which also contribute to the immune response against different types of microbial infections.6,8,10

Interestingly, expression of daw and dpp in Bombyx mori is differentially regulated in hemocytes of larvae infected with the nucleohedrovirus BmNPV, and virus replication can be reduced by overexpressing daw and dpp, or it can increase by RNAi knockdown of these molecules in B. mori culture cells.38 In addition, functional studies involving experiments to investigate the cellular immune response39 of larvae with mutations in certain TGF-β signaling molecules (including the type II receptors Punt and Wishful Thinking that can function in both the BMP and activin pathways) as well as the interaction between BMP/Activin signaling and the phenoloxidase/melanization activity will include both larvae and adult flies of the model insect D. melanogaster as well as natural insect hosts together with a collection of nematode parasites.11,16,40

We anticipate that results from these efforts will lead to better understanding of evolutionarily conserved mechanisms of the insect host anti-nematode immune defense. These are central questions that apply not only to insect/invertebrate models but also to the mammalian innate immune system and have parallels with other parasitic organisms; therefore, similar findings will contribute towards clarifying some of the underlying rules about how hosts regulate anti-nematode immune signaling.

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