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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

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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

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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 put 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.[21,25,28] 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 *Photorhabdus 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.[32,33]

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 $Pbac\{PB\}Mad^{c00574}$ 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 100 µl of 1.25% agarose. Infective juveniles were washed and adjusted to the final density of approximately 100 per larva, and 10 µl of nematode suspension was added to a single *D. melanogaster* larva. For uninfected controls, 10 µl 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

Table 1. List of primers and sequences used in quantitative RT-PCR experiments.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
rp49	GATGACCATCCGCCCAGCA	CGGACCGACAGCTGCTTGGC
diptericin	GCTGCGCAATCGCTTCTACT	TGGTGGAGTTGGGCTTCATG
actβ	CCATTCAAAGGCAGCAGGTG	AGCGGGTTGTGGAAATGACT
babo	CGCTCCATCTGGTGTAACGA	TCTGGTCCTTCGTCTTTGGC
daw	CGAGGAGGACGATGTACCGAT	GTGCTGCCTCTTGTGGATGA
dpp	TGGCGACTTTTCAAACGATTGT	CAGCGGAATATGAGCGGCAA
gbb	GGGACTCGGAATGGTTCTGC	CGTTGTCTATGTAAATCCCCGAC
mad	GACGAAGAGGAGAAGTGGGC	TAGATCACATGCGGCAGACC
myo	ATGCTGCGGTTGGAGAAAATA	CGTGACATATCGAGTTACACGG
sax	ACCCACACCTGCCAGAATG	CTTCCCCGTATTGCGTTTACT
smox	CGCCTATCAACAGCAACAGC	TGCCCACACTAAGCACACTC
scw	GCATCCTGGGCTCTGTGAAT	ACCGCAGCGTATCTGTCAAA

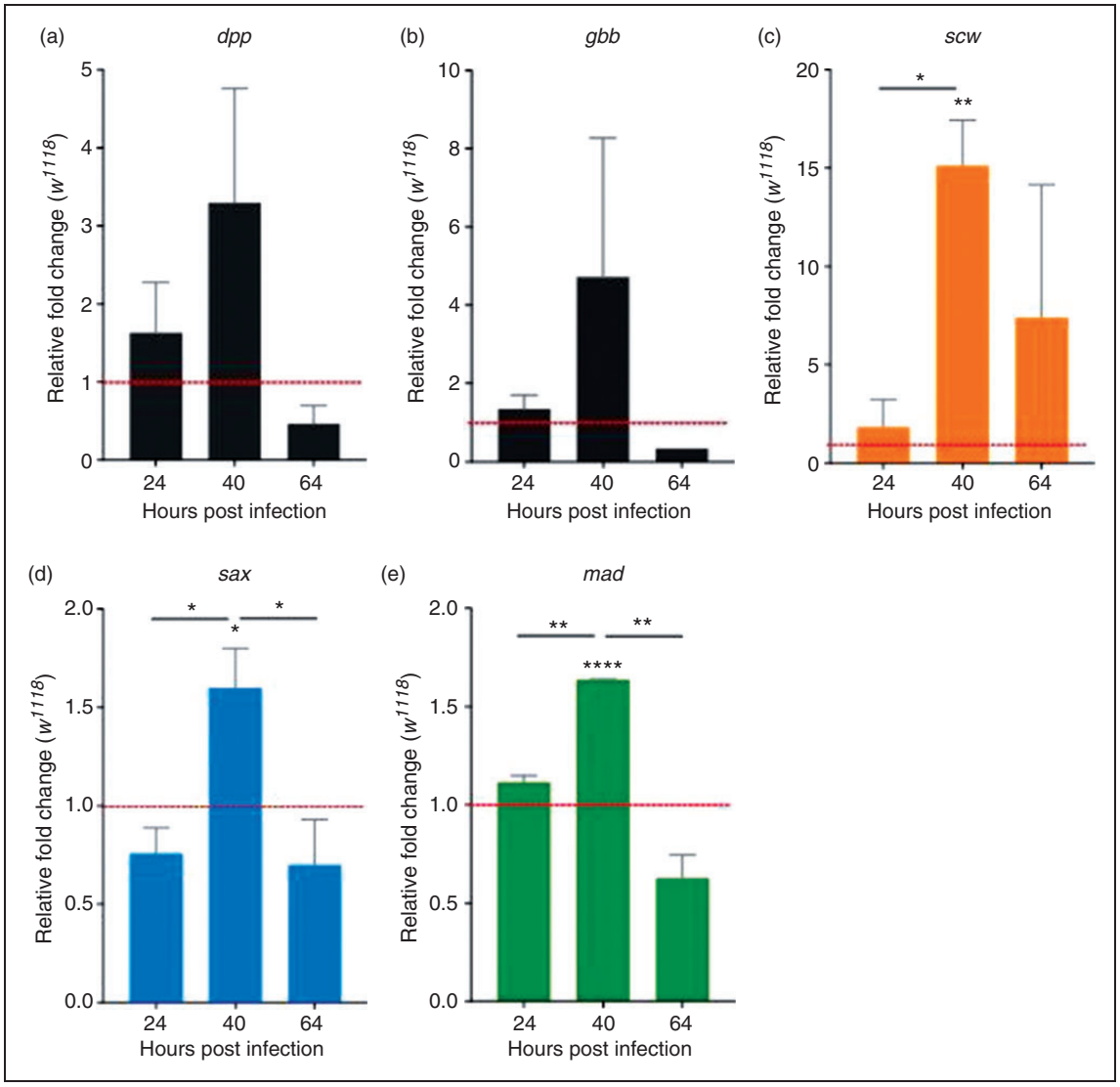

Figure 1. Within BMP signaling pathway, infection of *D. melanogaster* larvae with *H. gerrardi* nematodes leads to increased transcript levels of extracellular ligand *scw*, type I receptor *sax*, and transcription factor *mad*. Expression levels of extracellular ligands (a) *dpp* and (b) *gbb* are not significantly different from uninfected background control w^{1118} 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.

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 w^{1118} 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.

mad *is transcriptionally up-regulated via type I receptor babo of the activin pathway*

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 w^{1118} larvae at the mid timepoint (40 h) following infection with *H. gerrardi* compared with

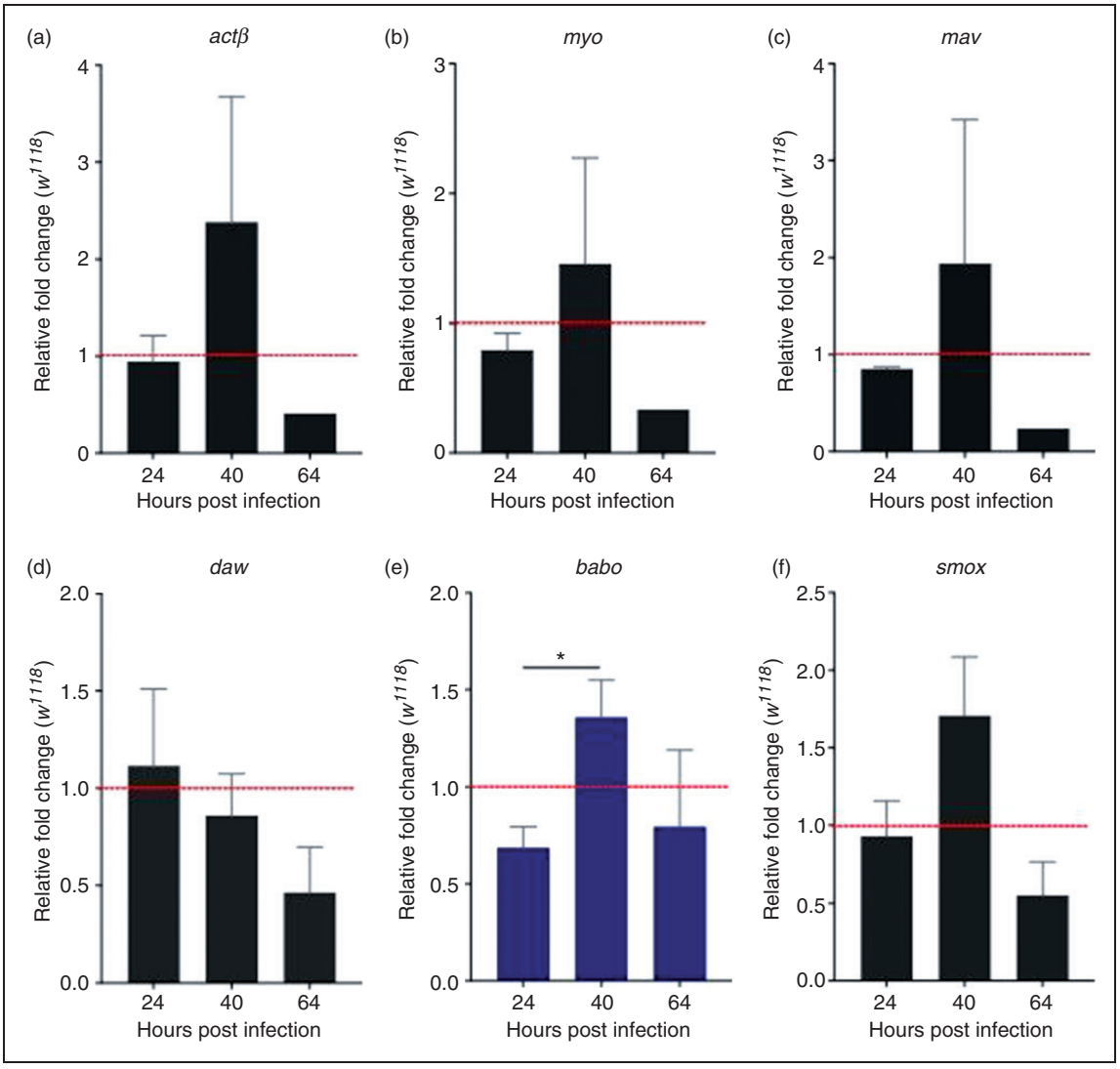

Figure 2. Within activin signaling pathway, infection of *D. melanogaster* larvae with *H. gerrardi* infective juveniles leads to increased transcript levels of receptor *babo*. The transcript levels of extracellular ligands (a) *actβ*, (b) *myo*, (c) *mav*, (d) *daw*, and (f) intracellular protein *smox* are not significantly different compared with the uninfected background control w^{1118} larvae at 24, 40, and 64 h post infection. (e) Transcript levels of *babo* are up-regulated at 40 h compared with 24 h post infection (***$P = 0.0379$). Red dotted line at 1 indicates normalization of fold change relative to uninfected controls.

uninfected controls (Figure 2e). In contrast, expression of intracellular protein *smox* downstream of *babo* was not affected by *H. gerrardi* nematode infection (Figure 2f). Collectively, these results suggest that the transcriptional changes of TGF-β signaling in *D. melanogaster* larvae upon *H. gerrardi* nematode infection are regulated via signal transducer Mad, through either of the signaling branches, the BMP pathway, or the activin pathway.

mad *up-regulation does not contribute to survival and antimicrobial peptide levels in* D. melanogaster *following infection with* H. gerrardi

Generally, the extent of Mad involvement in response against nematode infection is still unclear as the survival of *D. melanogaster mad* mutant larvae upon *H. gerrardi* nematode infection was not affected compared with their background control strain w^{1118} (Figure 3a). Furthermore, they only marginally triggered immune response, as read-out *diptericin* transcript levels were slightly higher, albeit insignificant, compared with w^{1118} (Figure 3b).

Our observation that transcript levels of *scw*, *sax*, and *mad* being up-regulated following infection with *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-β signaling in *D. melanogaster* larvae upon *H. gerrardi* attack are regulated via signal transducer Mad, through either of

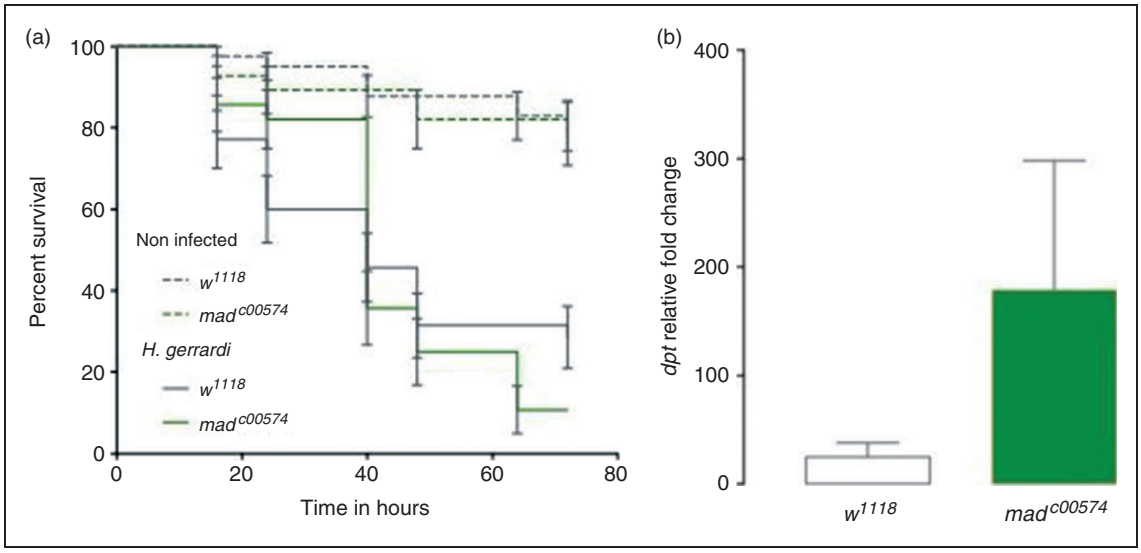

Figure 3. *Mad* inactivated *D. melanogaster* mutant larvae performed similarly as control larvae when challenged with *H. gerrardi* infective juveniles. (a) Survival of *Mad* mutant larvae was not significantly different compared with background control w^{1118} individuals. (b) At 24 h post infection, *diptericin* (*dpt*) transcript levels in *Mad* mutant larvae do not change significantly compared with w^{1118} background controls following *H. gerrardi* parasitic nematode infection.

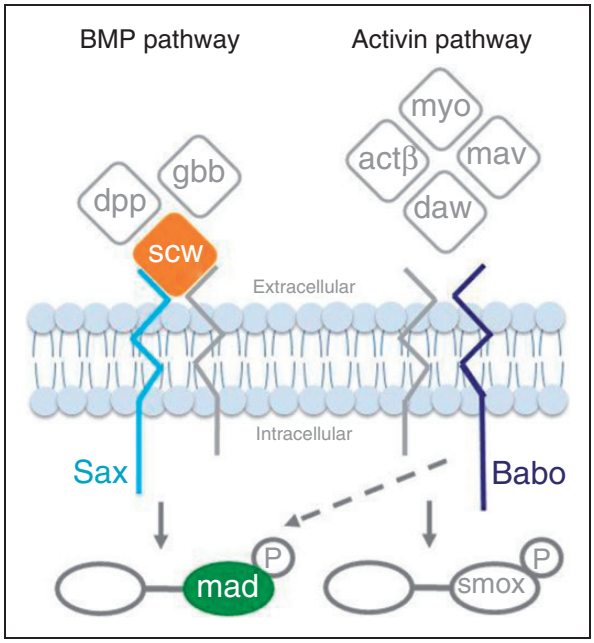

Figure 4. Proposed model for transcriptional regulation of TGF-β signaling pathway in *D. melanogaster* following infection with *H. gerrardi* parasitic nematodes. Upon infection with *H. gerrardi* infective juveniles, Mad up-regulation in *D. melanogaster* larvae can be achieved via both BMP and activin branches of the TGF-β signaling pathway. Type I receptors, Sax, and Babo, are up-regulated in response to nematode infection and can lead to up-regulation of Mad. Activation of type I receptor Sax is achieved via binding of the extracellular ligand Scw.

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 response[39] 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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