Published in final edited form as:

Immunobiology. 2016 December ; 221(12): 1362–1368. doi:10.1016/j.imbio.2016.07.011.

TGF- β signaling regulates resistance to parasitic nematode infection in *Drosophila melanogaster*

Ioannis Eleftherianos^{a,*}, Julio Cesar Castillo^{a,b}, and Jelena Patrnogic^a

^aDepartment of Biological Sciences, The George Washington University, Washington, DC, USA

^bLaboratory of Malaria and Vector Research, National Institutes of Health, MD, USA

Abstract

Over the past decade important advances have been made in the field of innate immunity; however, our appreciation of the signaling pathways and molecules that participate in host immune responses to parasitic nematode infections lags behind that of responses to microbial challenges. Here we have examined the regulation and immune activity of Transforming Growth Factor-beta (TGF- β) signaling in the model host *Drosophila melanogaster* upon infection with the nematode parasites *Heterorhabditis gerrardi* and *H. bacteriophora* containing their mutualistic bacteria *Photorhabdus*. We have found that the genes encoding the Activin and Bone Morphogenic Protein (BMP) ligands *Dawdle* (*Daw*) and *Decapentaplegic* (*Dpp*) are transcriptionally induced in flies responding to infection with the nematode parasites, containing or lacking their associated bacteria. We also show that deficient *Daw* or *Dpp* regulates the survival of *D. melanogaster* adults to the pathogens, whereas inactivation of *Daw* reduces the persistence of the nematodes in the mutant flies. These findings demonstrate a novel role for the TGF- β signaling pathways in the host anti-nematode immune response. Understanding the molecular mechanisms of host anti-nematode processes will potentially lead to the development of novel means for the efficient control of parasitic nematodes.

Keywords

Immunity; Infection; TGF- β

1. Introduction

Parasitic nematodes constitute one of the major threats to human health, causing diseases of major socioeconomic importance worldwide. Development of new control measures requires a better understanding of host factors that participate in the immune response against parasitic nematode infection. However, the lack of good animal models has limited investigations into mechanisms that hosts employ to oppose parasitic nematode attacks. The study of the interaction between invertebrate model hosts and nematode parasites provides

*Corresponding author at: Department of Biological Sciences, The George Washington University, 800 22nd Street NW, Washington, D.C., USA. ioannise@gwu.edu (I. Eleftherianos).

Conflict of interest

The authors declare that they have no competing financial interests.

insights into the processes underlying parasitic processes and host immunity, and complements the use of mammalian models by enabling whole-animal high throughput infection assays (Glavis-Bloom et al., 2012).

The fruit fly *Drosophila melanogaster* is well established as an outstanding model for studying innate immunity (Rämet, 2012). Elegant studies in *D. melanogaster* have led to the understanding of the NF- κ B immune signaling pathways Toll and Immune Deficiency (Imd) that are activated in response to microbial infections (Hetru and Hoffmann, 2009; Kleino and Silverman, 2014; Lindsay and Wasserman, 2014). Immunity research in *D. melanogaster* has further shown that the c-Jun N-terminal kinase (JNK) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathways also act in either competing or cooperative modes to regulate immune effector genes in the fly (Delaney et al., 2006; Myllymäki and Rämet, 2014). Therefore it is possible that additional or alternative evolutionary conserved pathways might regulate the activity of certain host immune functions against pathogenic infections (Castillo et al., 2011; Buchon et al., 2014).

Transforming Growth Factor-beta (TGF- β) family members contribute to a large variety of biological functions by acting through pathways with high degree of conservation at the sequence and functional level with regard to their vertebrate counterparts, the Activin and Bone Morphogenic Protein (BMP) signaling branches (Schmierer and Hill, 2007). TGF- β signaling pathways consist of extracellular ligands, a restricted number of transmembrane receptors, intracellular signal transducers and nuclear readout genes (Weiss and Attisano, 2013). Molecular and functional analysis of TGF- β signaling core components in model systems has the potential to provide a deep understanding of their activity and exact role in modulating a diverge range of physiological processes as well as controlling different steps of complex interactions within the organism (Li et al, 2006). In the fruit fly *D. melanogaster*, the Activin and BMP signals are transduced via receptor and Smad phosphorylation events that are essentially identical to those described for vertebrate TGF- β family signaling (Zi et al., 2012; Peterson and O'Connor, 2014). Interestingly, it was previously shown that TGF- β superfamily signals modulate the *D. melanogaster* immune response to wounding and bacterial infection (Clark et al, 2011).

The nematode parasite *Heterorhabditis gerrardi* contains the mutualistic bacteria *P. asymbiotica* that are able to infect both insects and humans (Plichta et al, 2009; Weissfeld et al., 2005). *Photorhabdus* bacteria produce a wide range of toxins, virulence factors and hydrolytic enzymes (Bode, 2009; Costa et al, 2010; French-Constant et al., 2007). Clinical isolates of *P. asymbiotica* have been recovered from patients in both the US and Australia (Gerrard et al., 2006; Wilkinson et al., 2010). In addition, the soil dwelling nematode parasite *H. bacteriophora* together with the bacteria *Photorhabdus luminescens* form a mutualistic complex that is pathogenic to insects (Ciche, 2007). *P. luminescens* bacteria are found in the gut of *H. bacteriophora* nematodes, which complete their life cycle in insect hosts (Waterfield et al., 2009). The *H. bacteriophora* infective juvenile stage is the only stage that is able to survive outside of the host and is required for insect infection (Ciche et al, 2006). Previous and recent work has demonstrated the power of using *D. melanogaster* for studying the molecular/genetic basis of insect immune responses against infections by entomopathogenic nematodes (Hallem et al., 2007; Wang et al., 2010; Hyrsl et al., 2011;

Dobes et al., 2012; Castillo et al., 2012, 2013; Arefin et al, 2014, 2015; Peña et al., 2015; Kucerova et al., 2016).

Here we have investigated the involvement of TGF- β signaling pathways in the *D. melanogaster* immune response against *H. gerrardi* and *H. bacteriophora* nematodes with or without their mutualistic *Photorhabdus* bacteria. We have found that the TGF- β ligands *Daw* and *Dpp* are transcriptionally activated in flies infected by *Heterorhabditis* and to a lesser extent by *Photorhabdus*, and that deficiencies in *Dpp* or *Daw* reduce the survival ability of the mutants in response to the nematodes. Characterization of the fly response to *Heterorhabditis* will potentially uncover novel evolutionary conserved host anti-nematode immune strategies.

2. Materials and methods

2.1. Fly stocks

The *D. melanogaster* strain *w*¹¹¹⁸ was used as background control in all experiments. Flies carrying a transposon insertion in *Daw* (strain d05680, Exelixis) and a spontaneous mutation *s1* in *Dpp* (strain 397, Bloomington) were used for infections. All fly strains were maintained and amplified for experimentation with approximately 2.5 g of Carolina Formula 4–24 Instant *Drosophila* media (Carolina Biological Supply), 10 mL of deionized water, and a dash of dry baker's yeast granules. All stocks were maintained at 25 °C and a 12:12-h light:dark photoperiod. Male and female adult flies 7–10 days old were used in infection assays with nematodes and bacteria.

2.2. Nematodes and bacteria

Heterorhabditis gerrardi and *H. bacteriophora* nematodes were amplified in fourth instar larvae of the wax moth *Galleria mellonella* using the water trap technique (White, 1927). Axenic nematodes were generated as previously described (Castillo et al., 2012; Eleftherianos et al, 2010b). To ensure that axenic *Heterorhabditis* nematodes were devoid of their associated *Photorhabdus* bacteria, the worms were homogenized and the nematode lysate was spread on agar plates. Fresh infective juveniles were collected and prepared through pelleting, washing and re-suspending in sterile distilled water (Castillo et al., 2012). *Heterorhabditis* infective juveniles with or without their corresponding *Photorhabdus* bacteria were used 1–2 weeks after collection from the water traps. For infections, nematode numbers were estimated by counting the average nematode density present in ten individual 50 μ L drops of water using a stereomicroscope.

The pathogens *Photorhabdus asymbiotica* (strain Kingscliff) and *P. luminescens* subsp. *Laumondii* (strain TT01) were used for fly infections. Bacteria were cultured in sterile 50 mL conical tubes containing 10 mL of Luria-Bertani broth and incubated for 18–24 h at 30 °C on a rotary shaker at 265 rpm. Bacterial cultures were centrifuged at 4°C, and the resulting pellets were washed and resuspended in sterile phosphate-buffered saline (PBS). The bacterial density of the suspension was estimated with an optical density measurement (600 nm), using a spectrophotometer (NanoDrop™ 2000c – Thermo Fisher Scientific) and a 10 $\times$ serial dilution plating technique.

2.3. Survival experiments

For nematode infections, *H gerrardi* and *H bacteriophora* nematodes were transferred into nested 5 mL cups (Solo, USA) containing filter papers (Whatman, USA) that supported 10 adult flies, as previously described (Castillo et al., 2012). A 1 mL solution containing approximately 1000 symbiotic or axenic *Heterorhabditis* nematodes was added to each container (100 infective juveniles/fly). Treatments involving sterile water only were used as negative controls. For bacterial infections, a PBS suspension (18.4 nl) containing cells of *P. asymbiotica* or *P. luminescens* was injected into the *D. melanogaster* hemocoel at the lateral anterior aspect of the thorax through nano-injection (Nanoject II – Drummond Scientific CO., USA). The number of *Photorhabdus* cells delivered into each fly was approximately 5×10^2 CFUs. PBS injections were used as negative controls. Injections with *Escherichia coli* and *Micrococcus luteus* were used as positive controls to activate the fly immune signaling. All fly infection assays were conducted between 10:00 AM and 12:00 PM. Flies infected by nematodes or bacteria as well as the uninfected controls were kept at 25 °C and survival experiments were conducted in triplicates.

2.4. Nematode load

Daw and *Dpp* mutants as well as background controls were infected with *H gerrardi* or *H bacteriophora* axenic or symbiotic nematodes, as described above. To estimate nematode load, flies were dissected at 24 h post infection and individual nematodes were counted using a tally counter and a stereomicroscope. Nematode load experiments were conducted three times and each assay involved the dissection of at least 15 individual flies infected with each type of nematode.

2.5. Gene transcription

Five adult flies from each experimental treatment were frozen at 3, 24 and 48 h post infection. Flies were homogenized using sterile plastic pestles and total RNA was extracted using the Pre-pEase RNA spin kit (USB) following the manufacturer's instructions. RNA samples were re-suspended in 40 µL of sterile nuclease-free water. RNA concentration was measured at an optical density of 260 nm using a Nanodrop (Thermo Scientific). Complementary DNA (cDNA) synthesis was carried out using the High Capacity cDNA reverse transcription kit (Applied Biosystems), random hexamers and 500 ng of RNA sample as starting material in a total reaction volume of 20 µL following the manufacturer's protocol. cDNA samples were diluted 1:10 in nuclease-free water and 1 µL was used as template for quantitative real-time RT-PCR (qRT-PCR) experiments using the EXPRESS SYBR GreenER kit with Premixed ROX (Invitrogen) and twin.tec real-time PCR 96-well plates on a Mastercycler ep realplex2 (Eppendorf). Primers for *Daw* (CG16987) and *Dpp* (CG9885) were purchased from Eurofin MWG Operon and the sequences and reaction conditions have been published before (Clark et al., 2011). For each sample, the amount of *Daw* or *Dpp* mRNA detected was normalized to mRNA values of the control housekeeping gene *RpL32* (CG7939) (Castillo et al., 2013). Normalized data were used to quantify the relative level of *Daw* or *Dpp* mRNA according to cycling threshold analysis (DCt), hence data were expressed as the ratio $2^{CT(RpL32)/2^{CT(Gene)}}$. The data are presented as a ratio between infected versus uninfected flies (negative controls).

2.6. Statistical analysis

All values were expressed as means $\pm$ standard deviation. Statistics were performed using the GraphPad Prism6 software. Data from the survival experiments were statistically analyzed using a Log-rank (Mantel-Cox) and Chi-square tests. P values below 0.05 were considered statistically significant. For gene transcription, data were analyzed using a one-way analysis of variance with a Tukey post-hoc test for multiple comparisons. Nematode load data were processed using unpaired two-tailed *t*-test.

3. Results

3.1. *Daw* and *dpp* are upregulated in *D. melanogaster* upon infection with the pathogens

We used qRT-PCR to estimate the transcript levels of *Daw* and *Dpp* in *w*¹¹¹⁸ adult flies at three time-points post infection with axenic or symbiotic *H. gerrardi* or *H. bacteriophora* nematodes, or injection with *P. asymbiotica* or *P. luminescens* bacteria. We also used untreated flies (baseline controls), flies injected with sterile PBS (injury controls), and flies injected with *E. coli* (Gram-negative) or *M. luteus* (Gram-positive) as inducers of the Imd and Toll signaling pathways, respectively. As previously shown (Clark et al., 2011), we found that *Daw* was significantly induced 24 h after infection with *M. luteus*, but not after infection with *E. coli* or injection with PBS (Fig. 1A). We also found high transcript levels of *Daw* in flies infected with symbiotic *H. gerrardi* and lower transcript levels in flies infected with axenic *H. gerrardi* or *P. asymbiotica* alone compared to flies injected with *E. coli* or *M. luteus* or to uninfected controls (Fig. 1A). We further observed that *Dpp* was transcribed at higher levels in flies infected with *H. gerrardi* or *P. asymbiotica* compared to control treatments, and that *Dpp* transcript levels were higher in flies infected with symbiotic nematodes than in those infected separately with axenic *H. gerrardi* or mutualistic *P. asymbiotica* (Fig. 1B). Similarly, we found higher *Daw* mRNA levels in flies infected with symbiotic *H. bacteriophora* compared to flies infected with axenic nematodes or *P. luminescens* only (Fig. 1C), and increased *Dpp* transcript levels in flies infected with axenic or symbiotic *H. bacteriophora* than in those injected with *P. luminescens* (Fig. 1D). These results indicate that TGF- κ signals are differentially regulated in *D. melanogaster* flies upon infection with parasitic nematodes and their mutualistic bacteria.

3.2. *Daw* loss-of-function mutant flies are sensitive to infection by nematodes and their bacteria

We assessed the ability of *Daw* and *Dpp* loss-of-function mutant flies to survive the infection of nematode parasites and their mutualistic bacteria, separately or together. We found that *Daw* mutants succumbed faster to infection with axenic *H. gerrardi*, symbiotic *H. gerrardi*, or *P. asymbiotica* compared to background control flies (Fig. 2A). However, *Dpp* mutants were more susceptible to infection with axenic or symbiotic *H. gerrardi*, but not to infection with *P. asymbiotica* alone, compared to controls (Fig. 2B). We also found that *Daw* mutant flies were more sensitive to axenic and symbiotic *H. bacteriophora* nematodes as well as to *P. luminescens* bacteria than the controls (Fig. 2C). Interestingly, *Dpp* mutants were more susceptible to infection with axenic *H. bacteriophora*, but not to infection with symbiotic nematodes or *P. luminescens* alone, than the control flies (Fig. 2D). Infections with *E. coli* and *M. luteus* did not affect the survival of TGF- β mutants compared to background controls

(Fig. S1). These results demonstrate that *Daw* and *Dpp* activity can modulate the survival of *D. melanogaster* flies to infection with parasitic nematodes and their mutualistic bacteria.

3.3. *Daw* mutants support nematode persistence

We then determined the numbers of nematode parasites contained in *Daw* and *Dpp* mutant flies and their background controls. We found that *Daw* mutants contained significantly higher numbers of axenic or symbiotic *H. gerrardi* (Fig. 3A) and *H. bacteriophora* (Fig. 3B) nematodes compared to control flies one day after infection with the parasites. However, there were no significant differences in the nematode load between *Dpp* mutants and background controls (Fig. 3A, B). Interestingly, TGF- β mutant flies and control individuals contained similar numbers of axenic or symbiotic *Heterorhabditis* nematodes (Fig. 3A, B). These results imply that *Daw*, but not *Dpp*, acts as resistance regulator against infection by parasitic nematodes in *D. melanogaster* flies.

4. Discussion

Studying the activation of signaling pathways in animal models, such as *D. melanogaster*, upon infection with pathogenic organisms provides an efficient strategy for understanding the molecular events that take place in the host during the infection process and the interaction between certain signaling components with pathogen virulence factors (Bier and Guichard, 2012). *Heterorhabditis* parasitic nematodes together with *D. melanogaster* and other insect models have started to generate important information on host anti-nematode immune signaling and the genetic basis of factors that participate in anti-nematode immune function (Castillo et al., 2011). In *D. melanogaster* larvae, infection with the *H. bacteriophora*-*P. luminescens* complex, but not with the nematodes alone, activates the Toll and Imd pathways as shown by the induction of genes encoding antimicrobial peptides (Hallem et al., 2007). However, inactivating Toll and/or Imd immune signaling does not affect the ability of the mutant flies to survive infection by *H. bacteriophora*, implying the potential involvement of other signaling pathways in the response against parasitic nematodes.

TGF- β superfamily secreted factors perform crucial regulatory roles in both vertebrates and invertebrates because they are expressed ubiquitously in multiple tissues during animal development (Massagué, 2012). However, TGF- β expression levels are often elevated during parasitic nematode infections in mammals (Finlay et al., 2014). This could be attributed to host homeostasis to limit the pathological effects due to immune activation, nematode induction of host TGF- β , or production of TGF- β homologs by the parasites to interfere with host immunity (Johnston et al., 2016). The role of TGF- β superfamily signals in the immune response of *D. melanogaster* adult flies to wounding and bacterial infection has been explored previously (Clark et al., 2011). *Daw* and *Dpp* were shown to be differentially regulated by immune challenge and they perform certain immune functions in the fly. *Daw* is activated by the Toll pathway, it is downregulated by wounding, and it suppresses the melanization response following infection by *Listeria monocytogenes*. *Dpp* is activated by wounding and infection by *Micrococcus luteus*, and represses AMP expression following wounding and in the absence of infection. We have also recently provided preliminary

evidence that *Daw* and *Gbb*, which encodes the extracellular BMP ligand Glass bottom boat, are transcriptionally upregulated upon infection with *H. bacteriophora* nematodes (Castillo et al., 2013).

Here we have confirmed and extended previous findings in mammals and *D. melanogaster* by showing the involvement of two TGF- β ligands, *Daw* (which is distantly related to vertebrate activin ligands) and *Dpp* (the homolog of vertebrate BMP-2 and BMP-4) in the immune response of the fly against two species of the nematode parasite *Heterorhabditis*. We have shown that i) *Daw* and *Dpp* genes are strongly upregulated in *D. melanogaster* flies upon infection with *H. gerrardi* and *H. bacteriophora* nematodes carrying or lacking their related *Photorhabdus* bacteria, ii) *Daw* and *Dpp* loss-of-function mutant flies are sensitive to infection with either *Heterorhabditis* species, and iii) *Daw*, but not *Dpp*, mutants are able to harbor increased numbers of nematode parasites. These findings point out a key role for the participation of TGF- β signals in the regulation of the fly anti-nematode immune response.

Daw and *Dpp* are transcriptionally upregulated in pericardial nephrocytes, salivary glands and a subset of hemocytes in uninfected flies (Clark et al., 2011), and we have found that both ligands are strongly induced in flies infected with *Heterorhabditis* nematodes and in some cases with *Photorhabdus* bacteria. We suspect that *Daw* and *Dpp*, and probably other extracellular Activin and BMP ligands, are probably induced in certain hemocyte types that interact with the pathogens during the infection process (Au et al., 2004; Castillo et al., 2011; Eleftherianos et al., 2010a). However, we cannot exclude the possibility that TGF- β signals might be activated in other tissues, such as the midgut, which is one of the target tissues for *Photorhabdus* and possibly for their cognate nematode parasites (Silva et al., 2002; Castillo et al., 2011). Indeed, BMP components, including *Dpp* and *Gbb*, were recently shown to be strongly upregulated in the midgut of adult flies after oral infection with *Erwinia carotovora*, and depletion of *Dpp* in intestinal progenitor cells results in reduced fly viability upon *Pseudomonas entomophila* ingestion (Zhou et al., 2015). In addition, *Dpp* expression in enteroblasts is involved in regulating intestinal stem cells by modulating the activation of several signaling pathways in a non-autonomous manner, suggesting that BMP signaling participates actively in maintaining gut homeostasis in response to pathogenic infections.

We also suspect that the increased susceptibility to nematode infection and high nematode load could be due to reduced function of hemocytes (e.g. lower hemocyte numbers, reduced hemocyte viability, aggregation, nodule formation), which form the primary mediators of the insect immune response against parasites (Honti et al., 2014). Reduced hemocyte activity in *Daw* mutants could further result in impaired encapsulation response against the nematodes that would in turn increase the number of free parasites in the infected flies. Notably, *Dpp* mutants proved sensitive to *Heterorhabditis* infection, but they contain similar numbers of nematodes as the control flies. One explanation for this result could be that cellular immune functions are not essential for nematode persistence in the *Dpp* flies. Alternatively, defects in cellular immunity in *Dpp* mutants could be possibly counterbalanced by enhanced humoral immune activity that is mainly regulated by NF- κ B immune signaling (Ligoxygakis, 2013). Confirming or disproving these possibilities will be a major aspect of our future studies.

Since *Daw* and *Dpp* are upregulated in *D. melanogaster* wild-type flies upon infection with *Heterorhabditis* and *Photorhabdus* and they are secreted signals (Clark et al., 2011), they may function in a systemic manner to regulate target genes in certain fly tissues or interfere with the expression of genes that are controlled by specific immune signaling pathways. Therefore, future work will focus on determining the transcription levels of TGF- β ligands in various tissues of flies infected with axenic or symbiotic *Heterorhabditis* nematodes, or *Photorhabdus* bacteria alone. If *Daw*, *Dpp* or other TGF- β components are induced in the fly gut upon nematode infection, we will assess their role in regulating fly survival by interfering with their expression specifically in the gut and whether they contribute to tissue regeneration in response to the parasites. We will also examine the activation of immune pathways by interfering with BMP or Activin signaling in infected and uninfected flies and we will estimate TGF- β signaling regulation in fly mutants for components of immune pathways following infection with the pathogens.

Results from such studies, in combination with recently acquired information concerning factors in *D. melanogaster* with anti-nematode activity (Wang et al., 2010; Hyrs et al., 2011; Kucerova et al, 2016) and genome-wide transcriptome analysis of the *D. melanogaster* response to parasitic nematode infection (Arefin et al, 2014; Castillo et al, 2015), will undoubtedly provide critical knowledge on novel immune mechanisms against nematode parasites and clarify some of the underlying rules about how hosts regulate anti-nematode and antibacterial immune function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Department of Biological Sciences at GWU for critical reading of the manuscript. The research herein was supported by NIH grants 1R01AI110675-01 and 1R56AI110675-01.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.imbio.2016.07.011>.

Abbreviations

TGF-β	transforming Growth Factor-beta
BMP	Bone Morphogenic Protein
Dpp	decapentaplegic
Daw	Dawdle
NF-κB	nuclear factor-kappa beta
JNL	c-Jun N-terminal kinase

JAK/STAT Janus kinase/signal transducer and activator of transcription

PBS phosphate-buffered saline

References

- Arefin B, Kucerova L, Dobes P, Markus R, Strnad H, Wang Z, Hyrsi P, Zurovec M, Theopold U. Genome-wide transcriptional analysis of *Drosophila* larvae infected by entomopathogenic nematodes shows involvement of complement, recognition and extracellular matrix proteins. *J Innate Immun.* 2014; 6:192–204. [PubMed: 23988573]
- Arefin B, Kucerova L, Krautz R, Kranenburg H, Parvin F, Theopold U. Apoptosis in hemocytes induces a shift in effector mechanisms in the *Drosophila* immune system and leads to a pro-inflammatory state. *PLoS One.* 2015; 10:e0136593. [PubMed: 26322507]
- Au C, Dean P, Reynolds SE, ffrench-Constant R. Effect of the insect pathogenic bacterium *Photorhabdus* on insect phagocytes. *Cell Microbiol.* 2004; 6:89–95. [PubMed: 14678333]
- Bier E, Guichard A. Deconstructing host-pathogen interactions in *Drosophila*. *Dis Model Mech.* 2012; 5:48–61. [PubMed: 21979942]
- Bode HB. Entomopathogenic bacteria as a source of secondary metabolites. *Curr Opin Chem Biol.* 2009; 13:224–230. [PubMed: 19345136]
- Buchon N, Silverman N, Cherry S. Immunity in *Drosophila melanogaster*—from microbial recognition to whole-organism physiology. *Nat Rev Immunol.* 2014; 14:796–810. [PubMed: 25421701]
- Castillo JC, Reynolds SE, Eleftherianos I. Insect immune responses to nematode parasites. *Trends Parasitol.* 2011; 27:537–547. [PubMed: 21982477]
- Castillo JC, Shokal U, Eleftherianos I. A novel method for infecting *Drosophila* adult flies with insect pathogenic nematodes. *Virulence.* 2012; 3:339–347. [PubMed: 22546901]
- Castillo JC, Shokal U, Eleftherianos I. Immune gene transcription in *Drosophila* adult flies infected by entomopathogenic nematodes and their mutualistic bacteria. *J Insect Physiol.* 2013; 59:179–185. [PubMed: 22902989]
- Castillo JC, Creasy T, Kumari P, Shetty A, Shokal U, Tallon LJ, Eleftherianos I. *Drosophila* anti-nematode and antibacterial immune regulators revealed by RNA-Seq. *BMC Genomics.* 2015; 16:519. [PubMed: 26162375]
- Ciche TA, Darby C, Ehlers RU, Forst S, Goodrich-Blair H. Dangerous liaisons: the symbiosis of entomopathogenic nematodes and bacteria. *Biol Control.* 2006; 38:22–46.
- Ciche T. The biology and genome of *Heterorhabditis bacteriophora*. *WormBook.* 2007:1–9.
- Clark RI, Woodcock KJ, Geissmann F, Trouillet C, Dionne MS. Multiple TGF- β superfamily signals modulate the adult *Drosophila* immune response. *Curr Biol.* 2011; 21:1672–1677. [PubMed: 21962711]
- Costa SC, Chavez CV, Jubelin G, Givaudan A, Escoubas JM, Brehélin M, Zumbihl R. Recent insight into the pathogenicity mechanisms of the emergent pathogen *Photorhabdus asymbiotica*. *Microbes Infect.* 2010; 12:182–189. [PubMed: 20034588]
- Delaney JR, Stoven S, Uvell H, Anderson KV, Engström Y, Mlodzik M. Cooperative control of *Drosophila* immune responses by the JNK and NF-kappa B signaling pathways. *EMBO J.* 2006; 25:3068–3077. [PubMed: 16763552]
- Dobes P, Wang Z, Markus R, Theopold U, Hyrsi P. An improved method for nematode infection assays in *Drosophila* larvae. *Fly.* 2012; 6:75–79. [PubMed: 22614785]
- Eleftherianos I, ffrench-Constant RH, Clarje DJ, Dowling AJ, Reynolds SE. Dissecting the immune response to the entomopathogen *Photorhabdus*. *Trends Parasitol.* 2010a; 18:552–560.
- Eleftherianos I, Joyce S, ffrench-Constant RH, Clarke DJ, Reynolds SE. Probing the tri-trophic interaction between insects, nematodes and *Photorhabdus*. *Parasitology.* 2010b; 137:1695–1706. [PubMed: 20500922]
- Finlay CM, Walsh KP, Mills KH. Induction of regulatory cells by helminth parasites: exploitation for the treatment of inflammatory diseases. *Immunol Rev.* 2014; 259:206–230. [PubMed: 24712468]

- Gerrard JG, Joyce SA, Clarke DJ, ffrench-Constant RH, Nimmo GR, Looke DF, Feil EJ, Pearce L, Waterfield NR. Nematode symbiont for *Photorhabdus asymbiotica*. *Emerg Infect Dis*. 2006; 12:1562–1564. [PubMed: 17176572]
- Glavis-Bloom J, Muhammed M, Mylonakis E. Of model hosts and man: using *Caenorhabditis elegans*, *Drosophila melanogaster* and *Galleria mellonella* as model hosts for infectious disease research. *Adv Exp Med Biol*. 2012; 710:11–17. [PubMed: 22127881]
- Hallem EA, Rengarajan M, Ciche TA, Sternberg PW. Nematodes, bacteria, and flies: a tripartite model for nematode parasitism. *Curr Biol*. 2007; 17:898–904. [PubMed: 17475494]
- Hetru C, Hoffmann JA. NF-kappaB in the immune response of *Drosophila*. *Cold Spring Harb Perspect Biol*. 2009; 1:a000232. [PubMed: 20457557]
- Honti V, Csordás G, Kurucz É, Márkus R, Andó I. The cell-mediated immunity of *Drosophila melanogaster*: hemocyte lineages, immune compartments, microanatomy and regulation. *Dev Comp Immunol*. 2014; 42:47–56. [PubMed: 23800719]
- Hyrsl P, Dobes P, Wang Z, Hauling T, Wilhelmsson C, Theopold U. Clotting factors and eicosanoids protect against nematode infections. *J Innate Immun*. 2011; 3:65–70. [PubMed: 20948189]
- Johnston CJ, Smyth DJ, Dresser DW, Maizels RM. TGF- β intolerance, development and regulation of immunity. *Cell Immunol*. 2016; 299:14–22. [PubMed: 26617281]
- Kleino A, Silverman N. The *Drosophila* IMD pathway in the activation of the humoral immune response. *Dev Comp Immunol*. 2014; 42:25–35. [PubMed: 23721820]
- Kucerova L, Broz V, Arefin B, Maaroufi HO, Hurychova J, Strnad H, Zurovec M, Theopold U. The *Drosophila* chitinase-like protein IDGF3 is involved in protection against nematodes and in wound healing. *J Innate Immun*. 2016; 8:199–210. [PubMed: 26694862]
- Li MO, Wan YY, Sanjabi S, Robertson AKL, Flavell RA. Transforming growth factor- β regulation of immune responses. *Annu Rev Immunol*. 2006; 24:99–146. [PubMed: 16551245]
- Ligoxygakis P. Genetics of immune recognition and response in *Drosophila* host defense. *Adv Genet*. 2013; 83:71–97. [PubMed: 23890212]
- Lindsay SA, Wasserman SA. Conventional and non-conventional *Drosophila* toll signaling. *Dev Comp Immunol*. 2014; 42:16–24. [PubMed: 23632253]
- Massagué J. TGF β signalling in context. *Nat Rev Mol Cell Biol*. 2012; 13:616–630. [PubMed: 22992590]
- Myllymäki H, Rämetsä M. JAK/STAT pathway in *Drosophila* immunity. *Scand J Immunol*. 2014; 79:377–385. [PubMed: 24673174]
- Peña JM, Carrillo MA, Hallem EA. Variation in the susceptibility of *Drosophila* to different entomopathogenic nematodes. *Infect Immun*. 2015; 83:1130–1138. [PubMed: 25561714]
- Peterson AJ, O'Connor MB. Strategies for exploring TGF- β signaling in *Drosophila*. *Methods*. 2014; 68:183–193. [PubMed: 24680699]
- Plichta KL, Joyce SA, Clarke D, Waterfield N, Stock SP. *Heterorhabditis gerrardi* n. sp (Nematoda: Heterorhabditidae): the hidden host of *Photorhabdus asymbiotica* (Enterobacteriaceae: gamma-Proteobacteria). *J Helminthol*. 2009; 83:309–320. [PubMed: 19216823]
- Rämetsä M. The fruit fly *Drosophila melanogaster* unfolds the secrets of innate immunity. *Acta Paediatr*. 2012; 101:900–905. [PubMed: 22606959]
- Schmierer B, Hill CS. TGF β -SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol*. 2007; 8:970–982. [PubMed: 18000526]
- Silva CP, Waterfield NR, Daborn PJ, Dean P, Chilver T, Au CP, Sharma S, Potter U, Reynolds SE, ffrench-Constant RH. Bacterial infection of a model insect: *Photorhabdus luminescens* and *Manduca sexta*. *Cell Microbiol*. 2002; 4:329–339. [PubMed: 12067318]
- Wang Z, Wilhelmsson C, Hyrsl P, Loof TG, Dobes P, Klupp M, Loseva O, Mörgelin M, Iklé J, Cripps RM, Herwald H, Theopold U. Pathogen entrapment by transglutaminase—a conserved early innate immune mechanism. *PLoS Pathog*. 2010; 6:e1000763. [PubMed: 20169185]
- Waterfield NR, Ciche T, Clarke D. *Photorhabdus* and a host of hosts. *Annu Rev Microbiol*. 2009; 63:557–574. [PubMed: 19575559]
- Weiss A, Attisano L. The TGF β superfamily signaling pathway. *Wiley Interdiscip Rev Dev Biol*. 2013; 2:47–63. [PubMed: 23799630]

- Weissfeld AS, Halliday RJ, Simmons DE, Trevino EA, Vance PH, O'Hara CM, Sowers EG, Kern R, Koy RD, Hodde K, Bing M, Lo C, Gerrard J, Vohra R, Harper J. *Photorhabdus asymbiotica*, a pathogen emerging on two continents that proves that there is no substitute for a well-trained clinical microbiologist. *J Clin Microbiol.* 2005; 43:4152–4155. [PubMed: 16081963]
- White GF. A method for obtaining infective nematode larvae from cultures. *Science.* 1927; 66:302–303.
- Wilkinson P, Paszkiewicz K, Moorhouse A, Szubert JM, Beatson S, Gerrard J, Waterfield NR, ffrench-Constant RH. New plasmids and putative virulence factors from the draft genome of an Australian clinical isolate of *Photorhabdus asymbiotica*. *FEMS Microbiol Lett.* 2010; 309:136–143. [PubMed: 20584081]
- Zhou J, Florescu S, Boettcher AL, Luo L, Dutta D, Kerr G, Cai Y, Edgar BA, Boutros M. Dpp/Gbb signaling is required for normal intestinal regeneration during infection. *Dev Biol.* 2015; 399:189–203. [PubMed: 25553980]
- Zi Z, Chapnick DA, Liu X. Dynamics of TGF- β /Smad signaling. *FEBS Lett.* 2012; 586:1921–1928. [PubMed: 22710166]
- ffrench-Constant RH, Dowling A, Waterfield NR. Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. *Toxicon.* 2007; 49:436–451. [PubMed: 17207509]

Fig. 1.

Daw and *Dpp* mRNA levels are up-regulated in *Drosophila melanogaster* adult flies in response to infection with *Heterorhabditis* nematodes. Transcript levels of *Daw* (A, C), and *Dpp* (B, D) in *D. melanogaster*¹¹¹⁸ adult flies at 3, 24 and 48 h following infection with *Heterorhabditis gerrardi* (A, B), axenic (Hg) or symbiotic (Hg + Pa) nematodes or *Photorhabdus asymbiotica* (Pa) bacteria alone, or after infection with *H. bacteriophora* (C, D) axenic (Hb) or symbiotic (Hb + PI) nematodes or *P. luminescens* (PI) bacteria only. Injection with *Escherichia coli* (Ec) and *Micrococcus luteus* (MI) were used as positive controls for the induction of immune signaling. *Daw* and *Dpp* transcriptional up-regulation is shown as relative abundance of transcripts normalized to *RpL32* and expressed as a ratio compared to flies ($n = 3$ individuals per experimental condition) injected with sterile PBS (negative control). Data analysis was performed by unpaired two-tailed t-test (GraphPad Prism6 software) and significant differences are indicated with asterisks (* $P < 0.05$, ** $P < 0.01$). NS

denotes non-significant differences between experimental treatments. Bars show the means from three independent experiments and error bars represent standard deviations.

Fig. 2.

Drosophila melanogaster *Daw* and *Dpp* deficient flies are sensitive to infection with *Heterorhabditis* nematodes. Survival of 7–10 day old loss-of-function mutants for *Daw* (A, C) and *Dpp* (B, D) following infection with (A, B) *Heterorhabditis gerrardi* axenic (Hg) or symbiotic (Hg + Pa) nematodes or intrathoracic injection with *Photorhabdus asymbiotica* (Pa) bacteria alone, or after infection with *H. bacteriophora* (C, D) axenic (Hb) or symbiotic (Hb + Pl) nematodes or injection with *P. luminescens* (Pl) bacteria alone ($n = 20$ flies per experimental condition). w^{1118} was used as the background strain (Control). Mutant and background flies treated with water only or injected with sterile PBS served as negative controls for nematode infections and bacterial injections, respectively. Survival was monitored for a week at daily intervals. Differences between survival curves were analyzed using Log-Rank test (GraphPad Prism6 software) and the values represent percent survival for the infected and uninfected flies. Significant differences are indicated with asterisks (* $P < 0.01$, ** $P < 0.001$). The means from three independent experiments are shown and error bars represent standard errors.

Fig. 3.

Drosophila melanogaster *Daw*, but not *Dpp*, deficient flies contain high numbers of *Heterorhabditis* nematodes. Numbers of *Heterorhabditis gerrardi* (A) axenic (Hg) and symbiotic (Hg + Pa) nematodes, *H. bacteriophora* (B) axenic (Hb) and symbiotic (Hb + PI) nematodes in 7–10 day old loss-of-function mutant flies for *Daw* and *Dpp* at 24 h following infection with the parasites ($n = 10$ –17 flies per experimental condition). w^{1118} was used as the background strain (Control). Differences in nematode load between the TGF- β mutants and the control flies were analyzed by unpaired two-tailed *t*-test (GraphPad Prism6 software) and significant differences are indicated with asterisks (* $P < 0.05$, ** $P < 0.01$). NS denotes non-significant differences between experimental treatments and horizontal bars show the mean values.